Vol. 38, No. 2 June, 1951

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

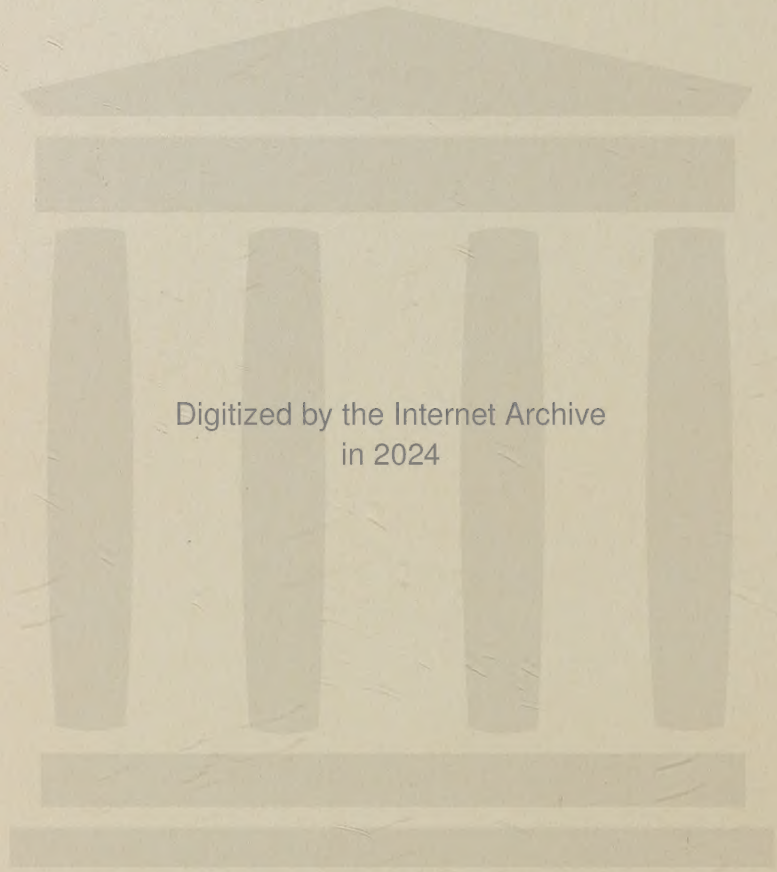
PUBLISHED BIMONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

PRINTED IN JAPAN



The Journal of Biochemistry, Vol. 38, No. 2.

ISOLIERUNG DER TETRAOXYNORSTEROCHOLANSÄURE AUS DER HÜHNERGALLE* UND ÜBER DIE GALLENSÄUREN DER GALLE VON CITELLUS MONGOLICUS RAMOSUS, THOMAS "HATARISU" UND VON SCHAFEN.**

Von KAZUMI YAMASAKI

(*Aus dem Biochemischen Institut der Medizinischen Abteilung der Tottori Universität, zu Yonago*)

(Der Schriftleitung zugegangen am 31 Januar, 1951)

In der Hühnergalle finden sich zwei spezifische Gallensäuren, Chenodesoxycholsäure und Cholsäure (*1*), wie es meistens bei der Vogelgalle der Fall ist (*2*). Ferner berichteten Hoshizima, u. a. (*3*), daß eine der Lithocholsäure isomere Gallensäure, Isolithocholsäure darin vorkomme.

Vor einigen Jahren wurde mir eine größere Menge von Hühnergalle von dem Apotheker Hayashi, zu Okayama geschenkt, wofür ich hier meinen herzlichen Dank aussprechen möchte. Die Galle wurde in der üblichen Weise hydrolysiert und nach der Entfettung durch $BaCl_2$-Fällung in zwei Fraktionen zerlegt. Wie im experimentallen Teil näher die Rede sein soll, wurde aus den beiden Fraktionen eine kleine Menge von Krystalle erhalten, die eine Hammarsten-Yamasakische Reaktion (*4*) gab, dagegen bei der Myliusschen Reaktion durch einen bräunlich-roten Farbenumschlag gekennzeichnet wurde, was bei der Cholsäure nicht der Fall ist. Die Reinigung dieser Krystalle war mit Schwierigkeiten verbunden und erst nach umständlicher Behandlung gelang es mir, daß sie mit der Tetraoxynorsterocholansäure $C_{27}H_{46}O_6$ identifiziert wurden, die zuerst von Ohta (*5*) aus der Galle von „Gigi"-fisch (Knochenfisch) isoliert und deren Konstitution durch nachherige Untersuchung von Isaka (*6*) endgültig als 3, 6, 12, 24-Tetraoxynorsterocholansäure (C_{27}) geklärt wurde.

Nach Shimizu (*7*) soll die Gallensäure im tierischen Organismus aus den mit der Nahrung zugeführten D-Provitamine sowie D-Vitamine

* Der Inhalt dieses Manuscriptes wurde bei der Versammlung der Biochemischen Gesellschaft West-Japans am 17. November 1948 auszügsweise berichtet.

** Diser Teil des Manuscriptes wurde in "Yonago-Igaku-Zasshi" **2**, 26 (1950) auf Japanisch publiziert.

gebildet werden und zwar wurde aus dem Okayama Institut eine Anzahl von höheren Gallensäuren in der Galle verschiedener Tiere berichtet (*7*), die als die Zwischenprodukte zwischen der gewöhnlichen C_{24}-Gallensäuren und den genannten Steroide betrachtet worden sind. Es ist sehr interessant, daß eine höhere Gallensäure wie die hier berichtete Gallensäure auch aus der Warmblütergalle entdeckt wurde und zwar um so mehr, weil die bisherig berichteten höheren Gallensäuren meistens aus der Galle von Krltblütern isoliert wkrden. Hervorzuheben ist auch, daß eine neue höhere Gallensäure von der Formel $C_2 H_4 O_c$, die mit der hier berichteten Säure isomer ist, gleichzeitig von Minato und Ikeda (*8*) aus der Galle von einem Warmblüter, „Todo" (Seetier) gefunden wurde.

Fine Art von Eichhörnchen, „Hatarisu" (*Citellus mongol'cus ramosus*, Thomas) soll eine charakteristische Empfindlichkeit gegen die Erreger der epidemischen Krankheiten des Menschen, insbesondere gegen die der Pest, die des Flecktyphus u. a. zeigen (*9*). Weil die Galle das Sekret der Leber ist, die das wichtigste Entgiftungsorgan des Organismus darstellt, so ist eine Besonderhe't der Gallenbestandteile dieses Tieres zu erwarten.

72.8 g. der Trockensubstanz dieser Galle wurden untersucht und das Taurocholsäure-Natrium krystallinisch erhalten und zwar machte es den Hauptbestandteil der Galle aus.

Hervorzuheben ist, daß die Gallensäure dieser Galle, wie unten näher die Rede sein soll, ausschliesslich aus Cholsäure bestand und keine Gallensäure der Desoxycholsäure-reihe gefunden wurde.

In der Galle von Schafen und von Ziegen wurden Cholsäure und Desoxycholsäure (nas Mengenverhältnis von 10 : 1) von Schenk (*10*) als Hauptgallensäuren dargestellt. Aus 2 Liter Schafgalle wurde neben diesen Hauptgallensäuren auch eine minimale Menge von Chenodesoxycholsäure als Formylester isoliert.

Wie durch die bisherigen Untersuchungen der Galle verschiedener Tierarten gezeigt wurde, stellt die Chenodesoxycholsäure eine große Verbreitung nächst der Cholsäure in der tierischen Welt dar.

EXPERIMENTELLER TEIL

Hühnergalle—Ca. 600 ml. Hühnergalle wurden mit 60 g. KOH 2 Tage lang gekocht und mit verdünnter Salzsäure gefällt. Die teigigweiche Fällung wurde in verdünntem Alkohol gelöst und mit Gasolin einige Male geschüttelt, um die Lipoide zu entfernen. Die alkoholische Schicht

Schicht wurde mit Ammoniak schwach alkalisch gemacht, auf dem Wasserbade von Alkohol verjakt und darauf nach weiterem Zusatz von Ammoniak mit 10% iger $BaCl_2$-Lösung vollständig gefällt. Die Ba-Fällung (23 g.) bestand hauptsächlich aus Ba-Salz der Chenodesoxycholäure, deren Gesamtausbeute ca. 14.7 g. betrug.

Das Filtrat der Ba-Fällung wurde mit Salzsäure gefällt und die getrocknete Fällung (4.5 g.) wurde mit absolutem Alkohol digeriert und daraus 0.9 g. eines reinen Cholsäurepräparates erhalten.

Isolierung der Tetraoxynorsterocholansäure.

Die oben erwähnte rohe Ba-Fällung (23 g.) wurde mit Sodalösung gekocht und nach der Entfernung der dabei entstandenen $BaCO_3$-Fällung mit Salzsäure gefällt. Die getrocknete Fällung (15 g.) wurde in Na-Methylat gelöst und mit der gleichen Menge von 10%iger $BaCl_2$-Lösung versetzt und auf dem Wasserbade erwärmt, wobei sich reichliche Krystalle abschieden. Das so erhaltene Ba-Salz der Chenodesoxycholsäure betrug 14.7 g. Das Filtrat wurde weiter eingeengt und ein weiteres Krystallisat erhalten.

Das von dem Krystallisat abfiltrierte Filtrat wurde weiter eingeengt, wobei sich ein Krystallisat mit amorpher Masse abschied, und durch abermalige Einengung eine weitere derartige Fraktion erhalten wurde. Die beiden Fraktionen der Krystalle wurden vereinigt, mit Sodalösung zerlegt und dann mit Salzsäure gefällt. Die getrocknete gelbe Fällung (0.2 g.) zeigte eine Hammarsten-Yamasakische Reaktion, aber der Farbenumschlag der Myliusschen Reaktion war nicht grün, sondern bräunlichrot. In einer kleinen Menge von Eisessig wurde sie gelöst, mit Wasser versetzt, wobei sie zur Gallert erstarrte und abgesaugt wurde. Diese Umfällung wurde einige Male wiederholt, um ein Krystallisat zu erhalten. Es schmolz unscharf bei 212-215°, wurde in verdünntem Alkohol gelöst, und darauf auf dem Wasserbade eingeengt, bis eine Krystallisation auftrat. Durdh Wiederholung dieser Umkrystallisierung wurde eine reine Säure erhalten. Schmelzpt. 216-217° (Sintern bei 215°). Keine Schmelzpunktdepression mit der reinen Tetraoxynorsterocholansäure)* aus „Gigi"-fischgalle. Myliussche Reaktion: bränulich-rot. Hammarsten-Yamasakische Reaktion: violettblau.

3.500 mg. Subst.: 8.887 mg. CO_2, 2.950 mg. H_2O.

$C_{27}H_{46}O_6$	Ber.	C	69.47	H	9.94
	Gef.	,,	69.25	,,	9.43

* Dieses Präpart wurde von Prof. T. Shimizu, Okayama freundlicherweise geschenkt, wofür ich herzlich danken mochte.

Aus dem oben erwähnten Filtrat der Ba-Fällung (Cholsäurefraktion) wurde auch eine kleine Menge von dieser höheren Gallensäure erhalten. Die Reinigung wurde sehr schwierig und umständlich.

Die Ausbeute an diser Säure aus der beiden Fraktionen betrug insgesamt 39 mg.

Galle von Citellus Mongolicus—Der getrocknete Alkoholextrakt der Galle (72.8 g.) wurde in 500 ml. Wasser gelöst und unter Ansäuerung mit verdünnter Salzsäure mit Äther einige Male geschüttelt, um von den Lipoidsubstanzen zu befreien. Die so behandelte Gallenflüssigkeit wurde nach der Neutralisation mit $FeCl_3$-Lösung gefällt, wobei sich nur eine kleine Menge von Fällung abschied. Das Filtrat wurde mit Sodalösung schwach alkalisch gemacht und filtriert. Es wurde nach wiedermaliger Ansäuerung (HCl) im Kumagawa-Sudoschen Apparat mit Äther erschöpfend extrahiert. Erst nach dieser Behandlung wurde das Taurocholat durch Sättigung mit Kochsalz krystallinisch erhalten, wie es bei der Fistelgalle der Hunde der Fall war (*11*). Das Krystallisat wurde mehrmals genau nach dem Verfahren von Tanaka (*11*) umkrystallisiert und schmolz bei 231-232°. Die Gesamtausbeute betrug 22.5 g.

47.3, 32.6 mg. Subst.: 1.135, 0.834 mg. N (Mikrokjeldahl).
$C_{26}H_{44}O_7NSNa.2H_2O$ Ber. N 2.44
Cef. ,, 2.40, 2.47.

1 g. dieser Krystalle wurde in der gewöhnlichen Weise hydrolysiert und die so erhaltene freie Gallensäure wurde mit Cholsäure als identisch erwiesen. Methylester 157-58°.

Titration der Säure: 0.1817 g. Subst. (getrocknet) verbauchten 4.40 ml. *N*/10 NaOH (Phenolphtalein).
$C_{24}H_{40}O_5$ Ber. 408 Gef. 413.

Aus dem Hydrolysat wurde ein Krystallisat (0.08 g.) erhalten und mit Taurin identifiziert. Schmelzpt. 300°.

Die oben erwähnte Eisenfällung wurde mit Sodalösung zerlegt und mit KOH hydrolysiert, wobei eine Menge von Cholsäure (1.2 g.) und Glycin (als Äthylesterhydrochlorid), dagegen keine andere Gallensäure erhalten wurde.

Aus dem Daten möchte man schliessen, daß die Fraktion der Eisenfällung ausschliesslich aus der Glycocholsäure bestand.

Schafgalle—1.90 Liter Galle wurden mit der gleichen Menge von Alkohol versetzt, um die Muzinsubstanz zu befreien. Die von Alkohol fast vollständig verjagte Gallenflüssigkeit wurde nach der Extraktion mit

Äther,* in der üblichen Weise mit KOH hydrolysiert und darauf mit verdünnter Salzsäure gefällt. Die ausgefällte Fällung betrug 70.7 g.

Aus dieser Fällung wurde die Cholsäure und die Desoxycholsäure in der üblichen Weise (*12*) dargestellt und die Ausbeute beider Säuren betrug je 3.27% und 0.37% der Galle und zwar war fast gleich mit der von Schenk angegebenen.

Der von diesen Gallensäuren befreite Ätherextrakt der Galle wurde von Äther verjakt, in verdünntem Ammoniak gelöst und darauf mit Alkohol und 10%iger $BaCl_2$-Lösung versetzt. Diese Mischung wurde auf dem Wasserbade erwärmt, wobei sich eine von Krystallen durchsetzte Fällung abschied. Die so erhaltene Fällung wurde mit Sodalösung zersetzt und mit verdünnter Salzsäure gefällt. Die Fällung betrug 0.6 g.

Sie wurde mit frisch destillierter Ameisensäure 40 Minuten lang gekocht und dann in kaltes Wasser gegossen. Die getrocknete Fällung wurde in kleiner Menge von Methanol gelöst und mit Wasser versetzt, wobei eine von Krystallen durchsetzte harzige Masse abgesetzt wurde. Die oben stehende Flüssigkeit wurde abdekantiert und dann mit kleiner Menge von Methanol digeriert. Die so erhaltenen Krystalle betrugen 0.1 g. Umkrystallisation aus Methanol-Wasser. Schmelzpt. 182–183° (Sintern 181°). Keine Schmelzpunktdepression mit dem reinen Chenodesoxycholsäureformylester. Liebermannsche Reaktion: bräunlichviolett.

4.065 mg. Subst.: 10.350 mg. CO_2, 3.200 mg. H_2O.

$C_{26}H_{40}O_6$ Ber. C 69.59 H 8.99

Gef. „ 69.44 8.81 „

ZUSSMMENFASSUNG

Die Galle von Hühner, von *Citellus mongolicus ramosus*, Thomas und von Schafen wurde untersucht und die Ergebnisse sind folgende:

1. Aus der Hühnergalle wurde die höhere Gallensäure $C_{27}H_{46}O_6$ isoliert, die zuerst aus der Galle von „Gigi"-fisch (Knochenfisch) dargestellt wurde.

2. Das taurocholsaure Natrium machte den Hauptbestandteil der Galle von *Citellus mongolicus ramosus*, Thomas aus.

3. Aus der Schafgalle wurde ausser den Hauptgallensäuren, Cholsäure und Desoxycholsäure auch eine minimale Menge von Chenodesoxycholsäure isoliert.

* Aus dem Ätherextrakt wurde Cholesterin (0.2 g.) erhalten.

LITERATUR

(*1*) Windaus, A. U. und Schoor, A. v., *Z. physiol. Chem.*, **161**, 143 (1926); Yamasaki, K., *J. Biochem.*, **18**, 323 (1933)
(*2*) Shimizu, T., *Kagaku-Zikkengaku*, Abt. 2, 10, 405 (1941)
(*3*) Hoshizima, T., Takata, H., Shibuya, S., und Uraki, Z., *J. Biochem.*, **12**, 393 (1931)
(*4*) Yamasaki, K., *J. Biochem.*, **18**, 311 (1933)
(*5*) Ohta, K., *Z. physiol. Chem.*, **259**, 53 (1939)
(*6*) Isaka, H., *Z. physiol. Chem.*, **266**, 117 (1940)
(*7*) Shimizu, T., *J. Japanese Biochem. Soc.*, **20**, 118 (1948)
(*8*) Minato, K. und Ikeda, J., *Yakugaku-Zasshi*, **69**, 95 (1949)
(*9*) Lepene, P., *Comp. rend. Aca. Sci.*, **103**, 374 (1931); Kono, M., Takahashi, G. und Shoyama, S., *Saikingaku-Zasshi*, **463**, 670 (1943), Watanabe, S., *Manshu-Igakuzasshi*, **33**, 1903 (1940)
(*10*) Schenk, M., *Z. physiol. Chem.*, **145**, 95; **148**, 218 (1925)
(*11*) Tanaka, T., *Z. physiol. Chem.*, **220**, 39 (1933)
(*12*) Gattermann, L., *Praxis des organischen Chemikers*, Aufl. XIX 367 (1925)

The Journal of Biochemistry, Vol. 38, No. 2.

PYRUVATE AND α-KETOGLUTARATE IN BLOOD AND URINE. I. NORMAL VALUES AND THE EFFECTS OF EXERCISE AND THIAMINE OR RIBOFLAVIN INJECTION

By TAIJI SHIMIZU

(From the Department of Biochemistry, Niigata University School of Medicine, Niigata)

(Received for publication, Feb. 1, 1951)

There are many reports on the concentration of the pyruvic acid in blood and urine, in most of which the acid was determined by the method of Clift and Cook (1932) (*1*), in which pyruvate was determined as a bisulfite binding substance, or by Lu's method (*2*) or its modified methods, in which pyruvate was determined as dinitrophenylhydrazone. The specificity of these methods are not satisfactory as reported before (*3*), so that the determination was undertaken newly by the modified Friedemann-Haugen's method (*3*) the specificity of which was proved to be satisfactory.

As to the α-ketoglutaric acid content in blood and urine, reports are very few because of the lack of a good estimation method. Only Krebs (1938) (*4*) reported the values of a few cases. As the determination method of α-ketoglutaric acid has been devised by us in the previous paper (*5*), the estimation of its content in blood and urine was carried out by this method in the present study.

As pyruvic acid is an important intermediate metabolite of carbohydrate and has an intimate relation to lactic acid, there are many reports on the effect of exercise upon it. For example, Johnson *et al.* (1937) (*6*) observed the increase of pyruvate in blood and urine after a hard exercise. Bollman *et al.* (1939) (*7*) found that muscle was the site of the pyruvate formation during the exercise in the experiment with rats. Moteki *et al.* (1949) (*8*,*9*) investigated the process of the pyruvate increase in blood and urine after exercise under several conditions. As α-ketoglutaric acid is also one of the compounds which are related to the intermidiate metabolism of carbohydrate, its content in blood might be affected in any way when the combustion of carbohydrate becomes vigorous during exercise. Thus it seemed desirable to measure the change of the concentration of α-ketoglutaric acid as well

as pyruvic acid in blood, owing to the exercise.

It is well-known that thiamine pyrophosphate is concerned with the oxidation of pyruvic acid as a coenzyme. Therefore it is an interesting problem to observe the effect of thiamine upon the pyruvic acid in blood and urine after muscular exercise. Lu *et al.* (1939) (*10*) reported that light muscular work increased the pyruvate content of the blood of a thiamine-deficient man remarkably and the previous intravenous injection of thiamine lowered this increase. Bollman *et al.* (1939) (*7*) stated that the time necessary for the increased pyruvate content of muscle to return to the resting value was delayed on thiamine-deficient rats, and Moteki (1949) (*9*) stated that the increase of blood pyruvate of healthy men owing to exercise was lowered by the previous intravenous injection of thiamine.

α-Ketoglutaric acid is thought to be an intermediate metabolite of the pyruvate oxidation as a member of the tricarboxylic acid cycle of Krebs (*11*), and Krebs *et al.* (1940) (*12*) stated that thiamine was necessary to form α-ketoglutarate from pyruvate. Barron (1941) (*13*) stated that thiamine was related to the oxidation of α-ketoglutaric acid and Green *et al.* (1941-42) (*14*) stated that an enzyme, whose coenzyme was thiamine pyrophosphate, catalysed the decarboxylation of α-ketoglutaric acid. Therefore, it is also an interesting problem to observe the effect of thiamine on the change of the α-ketoglutaric acid content in blood after muscular exercise.

Has riboflavin no relation to the changes of the contents of pyruvate and α-ketoglutarate in blood ? The enzymes containing riboflavin phosphate are generally concerned with biological oxidation, and the reactions of Krebs' cycle are thought to need these enzymes. Therefore it is purposeful to examine the effect of riboflavin on the change of α-ketonic acid content of blood during exercise.

Based upon these considerations, the author determined at first the normal contents of pyruvic and α-ketoglutaric acids in blood and urine, and then observed the effects of exercise and of the injection of thiamine and riboflavin on these acids in blood.

METHODS

Healthy students and workers in the laboratory were examined. In the case of the determination of blood ketonic acids, the experiments were carried out more than 3 hours after the last meal and during the experiments the sitting state was maintained as calmly as possible. The

blood was withdrawn with minimum stasis from cubital veins. In the case of the determination of daily urine, the men, who were examined, engaged in their ordinary daily work and only drinking was prohibited during the collection of urine. Toluene was added to the collected urine as preservative. As for the exercise the running of 1,000 m. distance was imposed, the time needed being from 2 minutes, 48 seconds to 5 minutes, 35 seconds.

For thiamine injection "Metabolin" (1 ml.=3 mg. thiamine) was injected subcutaneously, and for riboflavin injection "Bisulase" (2 ml.=5 mg. riboflavin) was injected subcutaneously. Pyruvic acid was determined by a modified Firedemann-Haugen's method (*3*), and α-ketoglutaric acid was determined by the method described before (*5*).

RESULTS

α-Ketonic Acids in Blood—Determining the α-ketonic acids in blood of 34 samples of healthy men, 21 to 40 years of age, the following results were obtained: the highest value of pyruvic acid was 1.11 mg./dl., the lowest 0.59 ml./dl. and the average was 0.85±0.02 mg./dl. The highest value of α-ketoglutaric acid was 0.86 mg./dl., the lowest 0.11 mg./dl. and the average was 0.60±0.02 dl./dl.

α-Ketonic Acids in Urine—α-Ketonic acids in daily urine of 10 healthy men, 23 to 40 years of age, were determined. The highest of the daily excretion of pyruvic acid in urine was 32 mg., the lowest 15 mg. and the average was 21±1.7 mg. The highest concentration of pyruvic acid of daily urine was 1.65 mg./dl., the lowest 0.59 mg./dl. and the average 0.91 ±0.11 mg./dl. The highest of the daily excretion of α-ketoglutaric acid in urine was 59 mg., the lowest 19 mg. and the average 30±3.6 mg. The highest concentration of α-ketoglutaric acid of daily urine was 1.77 mg./dl., the lowest 0.13 mg./dl., and the average was 1.10±0.12 mg./dl.

The Effect of Exercise on α-Ketonic Acids in Blood—8 healthy men, 21 to 40 years of age, were tested. The running of 1,000 m. distance was imposed upon them. Just before the running, just after the running, 5, 15, 30, 60, and 120 minutes after the running, the α-ketonic acids in blood were determined (Table I). All 8 persons showed almost the same course of fluctuation. Blood pyruvate began to increase just after the exercise, got the highest increase (3.5 to 4.9 mg./dl.) 5 to 15 minutes after the exercise and returned to the resting value 60 to 120 minutes after the exercise. Blood α-ketoglutarate decreased for a while just after the

exercise, then increased remarkably 5 minutes after the exercise (0.9 to 2.3 mg./dl.) and returned to the resting value 15 minutes after the exercise. The averages of these values are shown by the curves of real line in Fig. 1.

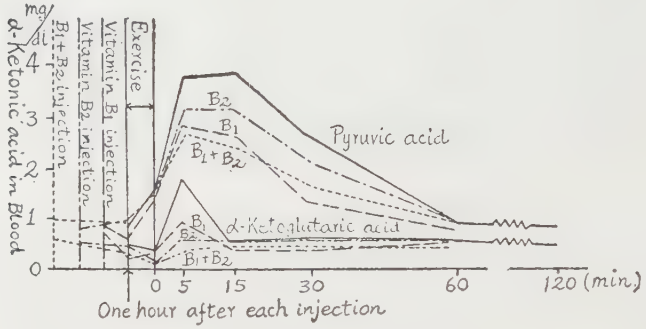


Fig. 1.

Table I

Name of person	Pyruvic acid in Blood (mg./dl.)						
	Before exercise	After exercise (Min.)					
		0	5	15	30	60	120
T. O.	0.98	0.76	4.89	3.93	3.03	0.97	1.00
T. N.	0.98	1.53	4.03	3.15	2.28	0.94	0.96
T. H.	0.94	1.57	3.80	4.58	2.96	0.99	
K. N.	0.94	1.49	3.85	4.92	3.00	0.96	0.97
K. O.	0.82	1.20	3.50	2.76	1.80	0.80	
Y. K.	0.82	1.91	3.38	4.07	2.47	0.89	0.80
T. A.	0.77	1.50	3.65	3.54	2.71	0.79	0.79
K. O.	0.73	1.51	3.20	3.85	2.79	0.95	0.75
Average	0.873 ±0.041	1.434 ±0.103	3.788 ±0.169	3.851 ±0.249	2.631 ±0.148	0.912 ±0.030	0.881 ±0.055

Name of person	α-Ketoglutaric acid in Blood (mg./dl.)						
	Before exercise	After exercise (Min.)					
		0	5	15	30	60	120
T. O.	0.69	0.41	1.99	0.65	0.56	0.65	0.61
T. N.	0.65	0.35	2.16	0.56	0.69	0.61	0.78
T. H.	0.48	0.04	2.33	0.48	0.56	0.43	
K. N.	0.60	0.17	1.90	0.56	0.56	0.60	0.52
K. O.	0.52	0.26	2.28	0.56	0.60	0.52	
Y. K.	0.11	0.26	1.30	0.35	0.26	0.26	0.30
T. A.	0.32	0.09	0.91	0.34	0.37	0.32	0.34
K. O.	0.43	0	1.17	0.49	0.45	0.41	0.37
Average	0.475 ±0.065	0.205 ±0.058	1.755 ±0.224	0.499 ±0.039	0.506 ±0.035	0.475 ±0.057	0.493 ±0.073

The Effect of Thiamine and Riboflavin Injections on α-Ketonic Acids in Blood of Resting Men—The same 7 or 8 healthy men as above were tested. 3 mg. of thiamine or 10 mg. of riboflavin, or 3 mg. of thiamine and 10 mg. of riboflavin at the same time were injected subcutaneously, and α-ketonic acids in blood were determined before and one hour after the injection (Table II). By the injection of 3 mg. of thiamine, pyruvate

TABLE II

3 mg. Thiamine Injection

Name	Pyruvic acid (mg./dl.)			α-Ketoglutaric acid (mg./dl.)		
	Before inj.	After inj.	Difference	Before inj.	After inj.	Difference
T. O.	0.70	0.49	−0.21	0.73	0.82	+0.09
T. N.	1.02	0.75	−0.27	0.63	0.21	−0.42
T. H.	1.00	0.66	−0.34	0.69	0	−0.69
K. N.	0.80	0.52	−0.28	0.52	0.04	−0.48
K. O.	0.79	0.52	−0.27	0.56	0.17	−0.39
Y. K.	1.10	0.91	−0.19	0.54	0.32	−0.22
T. A.	0.71	0.59	−0.12	0.63	0.17	−0.46
K. O.	0.91	0.54	−0.37	0.56	0.15	−0.51
Average	0.879 ±0.056	0.623 ±0.053	−0.263 ±0.020	0.608 ±0.030	0.235 ±0.079	−0.385 ±0.049

10 mg. Riboflavin Injection

Name	Pyruvic acid (mg./dl.)			α-Ketoglutaric acid (mg./dl.)		
	Before inj.	After inj.	Difference	Before inj.	After inj.	Difference
T. O.	0.81	0.82	+0.01	0.61	0.52	−0.09
T. N.	1.11	1.02	−0.09	0.61	0.43	−0.18
K. N.	0.86	1.03	+0.17	0.86	0.82	−0.04
K. O.	0.80	0.82	+0.02	0.41	0.28	−0.13
Y. K.	0.81	1.23	+0.42	0.45		
T. A.	0.73	0.89	+0.11	0.45	0.39	−0.06
K. O.	0.91	1.00	+0.09	0.48	0.37	−0.11
Average	0.869 ±0.041	0.973 ±0.057	+0.104 ±0.057	0.553 ±0.062	0.468 ±0.075	−0.102 ±0.022

3 mg. Thiamine+10 mg. Riboflavin Injection

Name	Pyruvic acid (mg./dl.)			α-Ketoglutaric acid (mg./dl.)		
	Before inj.	After inj.	Difference	Before inj.	After inj.	Difference
T. O.	1.02	1.03	+0.01	0.65	0.34	−0.31
T. N.	1.03	1.04	+0.01	0.67	0.41	−0.26
K. N.	0.92	0.91	−0.01	0.52	0.19	−0.33
K. O.	0.82	0.62	−0.20	0.60	0.41	−0.19
Y. K.	1.03	1.00	−0.03	0.63	0.45	−0.18
T. A.	0.76	0.64	−0.12	0.71	0.45	−0.26
K. O.	1.09	0.92	−0.17	0.43	0.19	−0.24
Average	0.953 ±0.052	0.880 ±0.073	−0.073 ±0.040	0.601 ±0.037	0.349 ±0.048	−0.253 ±0.022

and α-ketoglutarate in blood decreased together, by the injection of 10 mg. of riboflavin, blood pyruvate increased slightly but blook ketoglutarate decreased slightly, and by the injection of 3 mg. of thiamine and 10 mg. of riboflavin at the same time blood pyruvate showed mostly no change and blood ketoglutarate decreased. The average of these results is shown in the left part of Fig. 1.

The Effect of Thiamine and Riboflavin Injections on the Increase of α-Ketonic Acids in Blood after Exercise—One hour after the thiamine and riboflavin injections as in the previous experiment, the running of 1,000 m. distance

was imposed, and just before the injection, just before the running, just after the running, 5, 15, 30, and 60 minutes after the running the α-ketonic acids in blood were determined (Fig. 1). When 3 mg. of thiamine were injected, blood pyruvate began to increase just after the exercise, got the highest value 5 to 15 minutes after the exercise but showed much lower value than in the case of no vitamin injection, and returned to the resting value 60 minutes after the exercise. When 10 mg. of riboflavin were injected, blood pyruvate got the highest value 5 to 15 minutes after the exercise, but showed lower value than in the case of no vitamin injection and higher value than in the case of thiamine injection. When 3 mg. of thiamine and 10 mg. of riboflavin were injected at the same time, the increase of blood pyruvate after the exercise was repressed still more but showed almost the same tendency as in the case of thiamine injection.

When 3 mg. of thiamine were injected, blood ketoglutarate just after the exercise showed almost the same value as that before the exercise, and increased 5 minutes after the exercise but showed a much lower value than in the case of no vitamin injection. When 10 mg. of riboflavin were injected, blood ketoglutarate was descreased considerably just after the exercise and retured to the resting value 5 minutes after the exercise, and there was no increase after the exercise. When 3 mg. of thiamine and 10 mg. of riboflavin were injected together, blood ketoglutarate decreased still more. The averages of these results are shown in Fig. 1.

DISCUSSION

There are many reports on the normal value of blood pyruvate, but the results obtained by many investigators differ from each other remarkably. The values reported are, for instance, 0.55 mg. % by Lu (1939) (*2*), using his own method, 1.0 mg./dl. by Goldsmith (1949) (*17*), using Bueding-Worits' method (*18*), and 0.77 mg. % by Moteki *et al.* (1949) (*8*), using Friedemann-Haugen's method (*15*). Thus some investigators reported lower values than the values above obtained, and, generally speaking, normal pyruvate content of blood scarcely shows the value over 1 mg./dl. at rest and in fasting. As reported before (*3*), in determining the pyruvate content of urine, an error is apt to occur because of the lack of specificity of the method. Precise observation shows that the type of meals and of work during the collection of urine has influence upon the pyruvate excretion in urine. Moteki *et al.*

(1949) (*8*), using the method of Friedemann and Haugen (1943) (*15*), stated that the daily pyruvate excretion in urine of 8 workers in the laboratory were 4 to 6 mg. and those of railway workers were 10 to 248 mg. The author's results on workers in the laboratory showed a little higher values than those given by them. The author's estimation method is a modification of Friedemann-Haugen's (*15*). The chief point of the modification is to eliminate the effect of acetoacetate, and by this modification the specificity of the method has become almost complete. On the content of α-ketoglutaric acid of blood and urine, there is a report by Krebs (1938) (*4*). According to Krebs, the specimen from a case of polycythemia vera contained 1.05 mg. α-ketoglutaric acid in 100 ml. blood serum and the specimen from a case of cardiac decompensation 0.75 mg. in 100 ml. blood serum, and the excretion of α-ketoglutaric acid in daily urine of normal men was 15.5, 14.5 or 25.7 mg. As the figures in the present paper apparently show, a considerable amount of α-ketoglutaric acid exists in the normal blood and urine, and, moreover, the excretion of α-ketoglutaric acid in urine is larger than that of pyruvic acid.

As to the effect of exercise on the blood pyruvate, it is difficult to compare the results given by many investigators, as the time and intensity of exercises and the degree of fatigue are not the same in each report. Johnson *et al.* (1937) (*6*), Friedemann (1945) (*16*), Moteki (1949) (*9*) *etc.* observed that blood pyruvate began to be increased just after the exercise, got the maximum several minutes after the exercise and returned to the resting value scores of minutes after the exercise, and the present results coincide with these reports. But as the first example of Table I shows, sometimes the blood pyruvate is decreased slightly just after the exercise.

In the case when thiamine is injected previously, the increase of blood pyruvate after the exercise is repressed, possibly because of the decrease of pyruvate formation in muscles after the exercise, or because of the rapid treatment of pyruvate formed. As the enzymes containing thiamine are thought to be necessary for the treatment of pyruvic acid by tissues, the supply of thiamine may quicken the treatment of pyruvic acid formed after the exercise. The riboflavin injection represses the increase of blood pyruvate after exercise. Perhaps the flavin enzymes are also concerned with the oxidation of pyruvate.

We cannot find any communication as to the effect of exercise on the α-ketoglutaric acid in blood. Now the present experiment shows

that the blood ketoglutarate is decreased for a while just after the exercise, then begins to increase, gets the maximum value earlier than pyruvate and returns to the resting value also earlier than pyruvate. Also in this case, when thiamine is injected previously, the increase of ketoglutarate after exercise is repressed, and when riboflavin is injected, the action of repression is stronger than in the case of thiamine iejection, so that the increase of α-ketogluatric acid after the exercise does not occur. At any rate it is a noteworthy fact that thiamine and riboflavin have influence hot only upon pyruvic acid but also upon α-ketoglutaric acid.

SUMMARY

1. The normal value of pyruvic acid in the human blood was 0.85±0.02 mg./dl., and that of α-ketoglutaric acid was 0.60±0.02 mg./dl. The normal excretion of pyruvic acid in daily human urine was 21± 1.7 mg. and the concentration of the acid of daily urine was 0.91±0.11 mg./dl. The normal excretion of α-ketoglutaric acid in daily urine was 30±3.6 mg. and the concentration of the acid of daily urine was 1.10± 0.12 mg./dl.

2. After exercise both blood pyruvate and α-ketoglutarate was increased, and the latter returned to the normal value faster than the former.

3. One hour after the injection of 3 mg. of thiamine both pyruvate and α-ketoglutarate in blood were decreased. One hour after the injection of 10 mg. of riboflavin pyruvate in blood increased slightly and α-ketoglutarate decreased. One hour after the injection of both vitamins together, pyruvate did not show any remarkable change, whereas α-ketoglutarate was decreased.

4. The thiamine injection repressed the increase of both acids after exercise, and the riboflavin injection repressed the increase of α-ketoglutaric acid stronger than that of pyruvic acid. When both vitamins were injected together, the repression was the strongest.

The author wishes to express his sincere thanks to Prof. N. Shimazono for his guidance and helpful advice.

REFERENCES

(*1*) Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1783 (1932)
(*2*) Lu, C. D., *Biochem. J.*, **33**, 249 (1939)

(3) Shimizu, T., *J. Japanese Biochem. Soc.*, **22**, 108 (1950)
(4) Krebs, H. A., *Biochem. J.*, **32**, 108 (1938)
(5) Shimizu, T., *J. Biochem.*, **37**, 421 (1950)
(6) Johnson, R. E., and Ekwards, H. T., *J. Biol. Chem.*, **118**, 427 (1937)
(7) Bollmann, J. L., and Flock, E. V., *J. Biol. Chem.*, **130**, 565 (1939)
(8) Moteki, K., Kondo, T., and Suzuki, H., *Rinsho-Naika-Shonika*, **4**, 27 (1949)
(9) Moteki, K., *Seitai no Kagaku*, **1**, 28 (1949)
(10) Lu, C. D., and Platt, B. S., *Biochem. J.* **33**, 1538 (1939)
(11) Krebs, H. A., and Johnson, W. A., *Enzymol.*, **4**, 148 (1937)
(12) Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **34**, 1383 (1940)
(13) Barron, E. S. G., Goldinger, J. M., Lipton, M. A., and Lyman, C. M., *J. Biol. Chem.*, **141**, 975 (1941)
(14) Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **140**, 633 (1941); **145**, 69 (1942)
(15) Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943)
(16) Friedemann, T. E., *J. Biol. Chem.*, **157**, 673 (1945)
(17) Goldsmith, G. A., *Am. J. Med. Sci.*, **215**, 182 (1948)
(18) Bueding, E., and Wortis, H., *J. Biol. Chem.*, **133**, 585 (1940).

The Journal of Biochemistry, Vol. 38, No. 2.

PURIFICATION OF β-GLUCOSIDASE OF ASPERGILLUS NIGER II*

By KOYATA NIWA

(*From the Botanical Institute, Tokyo Bunrika University, Tokyo*)

(Received for publication, Feb. 5, 1951)

In a previous paper (*1*) it was shown that tannin precipitation method was a suitable one for the purification of the β-glucosidase of *Aspergillus niger* and that it might be also useful for separating this enzyme from accompanying carbohydrases. By the use of this procedure the purification of β-glucosidase has been effected as far as 8,000 folds with regard to the dried powder of the mould. Together with β-glucosidase this mould contains several other carbohydrases in varying amounts and among them saccharase in the most outstanding. Even the purest β-glucosidase preparation thus far obtained contained more than one fifth its amount of saccharase.

Accordingly for the next step of purification, it seemed desirable to remove saccharase as completely as possible, along with the elevation of the β-glucosidase activity.

The present work was undertaken with this aim, and some satisfactory results were obtained. Repeated precipetation of the enzyme with tannin followed by two folds adsorption on aluminum hydroxide gel B yielded a highly active β-glucosidase preparation, which is about 10,000 times as active as the dried powder of the mould and contained but a minute quantity of saccharase and other oligases.

EXPERIMENTAL

β-Glucosidase Activity—β-Glucosidase activity of each preparation was determined with phenol-β-glucoside as snbstrate under the same experimental conditions as described previously and the specific activity was calculated in the same way (*1*, *2*).

Saccharase Activity—Saccharase activity was estimated under the same conditions af for the measurement of β-glucosidase activity except with

* Present Study was supported by a grant in aid for Scientific Research from the Ministry of Education given to T. Miwa.

pH 4.0 (glycocoll-HCl buffer), which is optimal for the saccharase of this mould.

Preparation of β-Glucosidase Free from Saccharase—

Extraction: 800 g. of the dried mould with about 10 per cent of moisture were extracted with about 15 *l.* of water, covered with toluene, under continuous shaking at room temperature (20°). After about 5 hours, the mixture was filtered through a folded filter paper. The residue was washed repeatedly with small portions of water and the washings were combined to the filtrate. In this way 10 *l.* of the enzyme extract were obtained, which contained 103.3 β-glucosidase units* of the specific activity of *F*ph.-β-gl. 1.0. In this extract saccharase was about 3.7 times as active as β-glucosidase.

Tannin Precipitation: The principal procedure for the isolation of β-glucosidase from the crude enzyme extract consisted of a series of fractional precipitation with tannin. The results are indicated in the following table.

TABLE I

Step No.	Concn. of tannin	Repetition of tannin treatment	Volume	Total enzyme	Yield of enzyme	Specific activity	Activity ratio
	per cent	times	ml.	unit	per cent	*F*ph.-β-gl.	β-gl : sacch.
Starting extract			10.000	103.3	100.0	1.0	1 : 3.70
I	0.50	3	50	96.2	93.0	160.0	1 : 0.61
II	0.44	4	40	91.0	88.1	192.5	1 : 0.28
III	0.28	3	40	87.5	84.7	228.5	1 : 0.11
IV	0.20	3	40	80.0	76.9	365.0	1 : 0.064
V	0.44	3	40	76.3	73.3	424.0	1 : 0.016

Further repetitions of this treatment were not so effective.

Adsorption and Elution: For further purification of β-glucosidase adsorption method was employed. As adsorbent aluminum hydroxide gel B was used with success.

(A) To 16 ml. of the purified enzyme solution obtained by tannin

* The β-glucosidase unit was expressed as the amount of enzyme which brings about 50 percent hydrolysis of phenol-β-glucoside in 1 minute.

precipitation were added 5 ml. of aluminum hydroxide gel B (215.55 mg. as Al_2O_3). After keeping 5 minutes at room temperature, the mixture was centrifuged.

The adsorption of β-glucosidase was found to be almost quantitative, as the supertatant liquid contained only trace (0.03 per cent) of the enzyme.

1st Elution: the adsorbate was treated 3 times each with 10 ml. of 1 per cent solution of KH_2PO_4 and finally with 10 ml. of water. In the combined solution only 1 per cent of the enzyme was present.

2nd Elution: from the above residue β-glucosidase was eluted with in total 30 ml. of 1 per cent solution of Na_2HPO_4 in 6 portions, and the eluate was dialyzed against running water for about 48 hours.

Volume 30 ml.; *F*ph.-β-gl., 507, β-Glucosidase units 12.2 (Yield of the enzyme, 50 per cent); Activity ratio of β-glucosidase and saccharase 1 : 0.009.

(B) The same adsorption procedure as A was repeated with 25 ml. of the enzyme solution obtained above.

In this procedure again all of β-glucosidase in solution was adsorbed and only 2.8 per cent of the enzyme were found in the acid phosphate eluate. The main part of the enzyme could be eluated subsequently by 1 per cent solution of Na_2HPO_4 and dialyzed as above.

Volume 30 ml.; *F*ph.-β-gl., 1050; β-Glucosidase units 9.4 (Yield of the enzyme, 83 per cent of (A)); Activity ratio of β-glucosidase and saccharase, 1 : 0,0014.

As further repetition of this procedure resulted in the destruction of β-glucosidase, especially in the course of dialysis, the purification was stopped at this stage.

Properties of the Purest β-Glucosidase Preparation—

(A) Chemical Properties: The nitrogen content of the preparation was found to be 15.7 per cent (micro-Kjeldahl), suggesting the protein nature of this enzyme. On incineration no ash was left.

(B) Enzymatic Properties:

pH Relationship—Following figures illustrate the pH relationships of this enzyme preparation in the hydrolysis of 4 different β-glucosides. In all cases acidity optimum is found at about pH 3.0 and this value is in good accordance with that obtained with a crude preparation (*1*).

Relative Specificity—Table II shows data on the hydrolysis of various β-glucosides with different aglucons by this enzyme preparation.

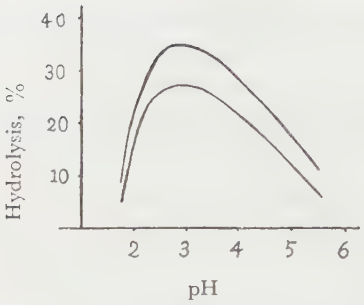


FIG. 1. Phenol-β-glucoside

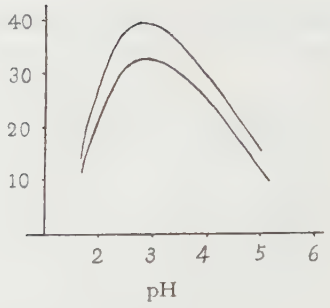


FIG. 2. Salicin

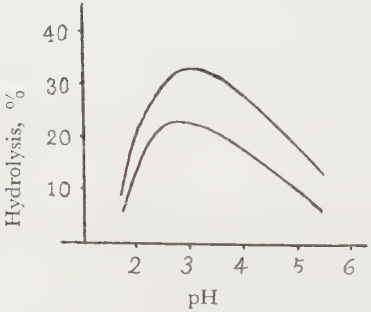


FIG. 3. *p*-Cresol-β-glucoside

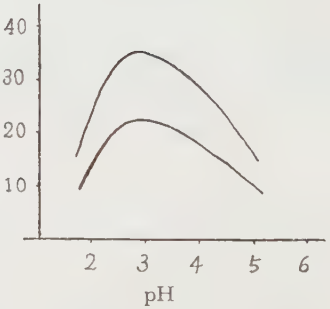


FIG. 4. *p*-Nitrophenol-β-glucosid

TABLE II

β-Glucoside	Dilution factor	Minutes	Hydrolysis in per cent	$k \times 10^1$	Mean	$k \times 10^1 \times$ Dil. fact.	Activity ratio
Phenol-	40	70	30.0	2.21	2.13	85.2	1.00*
		95	36.3	2.06			
		120	44.2	2.12			
Saligenin-	20	120	35.1	1.57	1.55	31.0	0.36
		150	42.2	1.53			
o-Cresol-	2	120	34.5	1.53	1.53	3.06	0.036
		150	42.2	1.52			
p-Cresol-	40	60	27.6	2.34	2.41	96.4	1.13
		120	43.5	2.43			
p-Nitrophenol-	20	50	22.1	2.17	2.17	43.4	0.50
		80	33.0	2.17			
Vanillin-	20	50	20.6	2.00	2.04	40.8	0.48
		80	31.7	2.07			

* The rate of hydrolysis of the phenol-β-glucoside is taken as unity.

The ratio of enzyme activity toward different substrates remained almost unchanged as compared with that previously observed with cruder enzyme preparations (*1*).

This is indicative of the fact that the relative specificity, as expressed in terms of activity ratio, is characteristic for β-glucosidase from respective sources and hardly affected by admixtures of the enzyme preparation.

Other Carbohydrases—Activity of some other carbohydrases of this β-glucosidase preparation was examined. As shown in Table III, this preparation is nearly free from oligases such as α-glucosidase, maltase, α- and β-galactosidase and the contamination of saccharase is also almost negligible

TABLE III

Substrate	pH*	Minutes	Hydrolysis in per cent	k×10³	Mean	Activity ratio
Phenol-β-glucoside	2.75	10 15 20	18.3 25.2 33.8	8.78 8.41 8.76	8.72×10** 87.2	1.00***
Phenol-α-glucoside	4.0	3000 6000	9.7 20.1	0.015 0.016	0.016	0.0002
Maltose-	4.0	3000 4000	12.9 17.1	0.020 0.020	0.026	0.0002
Phenol-β-galactoside	4.0	6000 10000	15.4 20.6	0.012 0.010	0.010	0.0001
Phenol-α-galactoside	4.0	4000 6000	26.4 35.7	0.033 0.032	0.033	0.0004
Saccharase	4.0	950 1100	23.5 26.4	0.122 0.122	0.122	0.0014

* Enzyme activity was measured at optimal pH of each glycosidase respectively.

** For the measurement of β-glucosidase activity the enzyme solution was diluted with water in proportion to 1 : 10.

*** Activity of β-glucosidase was taken as unity.

The problem of identity or nonidentity of β-glucosidase and β-galactosidase has often been a matter of controversy (*3*, *4*). The separate existence of these enzymes in Taka-diastase and in Aspergillus niger respectively, has been suggested in various ways (*5*, *6*, *7*). Present study offered for the first time unequivocal proof for the non-identity of these two enzymes in *Aspergillus niger*.

SUMMARY

1. From *Aspergillus niger*, β-glucosidase preparation with very high activity (*F*ph.-β-gl., 1050) was obtained by means of repeated tannin precipitation followed by adsorption with aluminum hydroxide gel B.

2. This preparation contained 15.7 per cent of nitrogen and no ash.

3. The preparation is almost free from oligases such as α-glucosidase, maltase, α- and β-galactosidase with a very minute content of saccharase.

The author wishes to express his gratitude to Prof. Tomoo Miwa for his constant advice and helpful criticism. Thanks are also due to Miss Sumiko Ishikawa for her technical assistance throughout this work.

REFERENCES

(*1*) Niwa, K., *J. Biochem.*, **37**, 301 (1950)

(*2*) Miwa, T., Cheng, C., Fujisaki, M., and Toishi, A., *Acta Phytochim*, **10**, 155 (1937)

(*3*) Helferich, B., *Ergeb. Enzymforsch.*, **2**, 74 (1933); **7**, 83 (1938)

(*4*) Neuberg, C., and Hofmann, E., *Biochem. Z.*, **256**, 450; **462** (1932); **267**, 209 (1933); **272**, 133 (1934)

(*5*) Nishizawa, K., *Sci. Rep. Tokyo Bunrika Daigaku*, B., **6** 43 (1942)

(*6*) Miwa, T., Nishizawa, K., Kinugawa, H. and Miwa, A., *Medicine and Biology*, **1**, 6 (1942) (In Japanese)

(*7*) Miwa T., and Miwa, A., *Medicine and Biology*, **2**, 396 (1942) (In Japanese).

The Journal of Biochemistry, Vol. 38, No. 2.

STUDIES ON PROTAMINE (III)

By YOSHIO KURODA

(*From the Department of Medical Chemistry, Kyushu University, Fukuoka*)

(Received for publication, Feb. 5, 1951)

Several chemical analyses of the amino acid composition of protamine by conventional methods have been presented, but the results are unsatisfactory to us because they give no decisive value.

Using partition chromatography and microbiological assay methods, the amino acids composition of protamines has been recently much elucidated by Block *et al.* (*1*, *2*), Tristram (*3*), Hamer and Woodhouse (*4*).

On account of its simple structure and small molecular weight, protamine has remained a favourite topic among protein chemists. In our laboratory, we have also carried out a series of study on protamine of various sources (*5*, *6*, *7*), and this report is dealing with our analytical results of protamine achieved by new device combining the copper method with paper partition chromatography.

EXPERIMENTAL

Reagents—(1) Copper phosphate suspension which is made by mixing the following substances (a), (b), and (c) in the ratio of 1 : 2 : 2 (*16*): (a) 27.3 g. of $CuCl_2$ in 1 *l.* of water; (b) 64.5 g. of Na_2HPO_4 and 7.2 g. NaOH in 1 *l.* of CO_2-free water; (c) 57.21 g. of Sodium borate and 100 ml. of 1 *N* HCl in 2 *l.* of water. (2) Sodium diethyldithiocarbamate solution (*16*, *14*): 0.2% aqueous solution of the carbamate reagent. To be used after filteration. (3) Carbon tetrachloride (*14*): To be purified by distillation. (4) Hydroxyl-amine solution (*14*): 4% aqueous solution of the pure material.

Procedure—The protamines were purified according to Kossel (*8*). The hydrolysis was carried out by heating the protamine in a small sealed tube (3 mm.×7 mm.) with 20% HCl for 10 hours. at 100°.

Two dimensional paper chromatogram was developed on 45 cm.× 45 cm. Toyoroshi No. 2 paper with phenol (or *n*-butanol—acetic acid mixture) and collidine—lutidine mixture as solvents. The positions of

amino acids were located by treating the paper with 0.025-0.05% ninhydrin in *n*-butanol. After heating at below 100°, the colored spots were cut our leaving a margin with stainless shears, and the residual solvent in each piece of the paper was rinsed out with dry ether. It was placed in a dry glass-stoppered Erlenmeyer-flask, treated with 15.0 ml. of the copper phosphate suspension and left stand for 6 hours. After filtration through a piece of Toroyoshi No. 131 paper (diam., 9 cm.), 10.0 ml. of the filtrate, namely the soluble copper-complex solution, was pipetted into another glass-stoppered test tube. Adding 0.5 ml. of the carbamate solution, 0.5 ml. of the hydroxylamine solution and 5.0 ml. of carbon tetrachloride, but tube was stoppered and shaken vigorously for about 30 seconds. The carbon tetrachloride layer was drawn up by means of a pipette and filtered into a dry tube through a dry Toyoroshi No. 1 paper in order to remove water.

The characteristic golden yellow color was determined with photoelectric colorimeter. We used an Electophotometer Hitachi (Type EPO-A) with cells 3.5 mm. in thickness and the filterglass (max. T.: 420 mμ).

Results—

(1) Tests with standard copper and amino acid solution: Each specimen was treated by the procedure mentioned above and the standard curves shown in Fig. 1 and 2 were obtained.

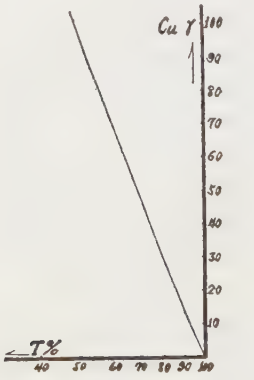


Fig. 1.

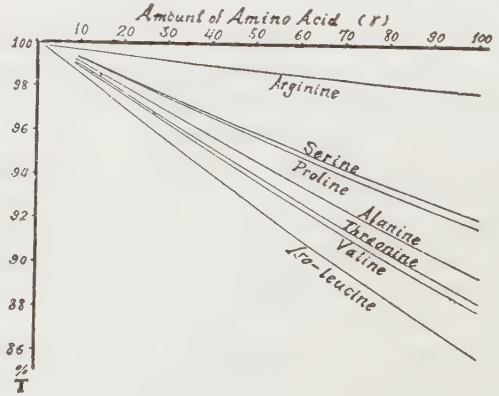


Fig. 2.

(2) Recovery test of amino acid: The acids were recovered as follows:—

Iso-leucine, serine 98%
Proline, arginine 97%
Valine, alanine, threonine 96%

(3) Amino acids of some protamines were determined on their hydrolysates by the above procedures. The results are shown in Table I.

TABLE I

Protamine	Clupeine		Scombrine		Spheroidine
Investigator / Amino acid	Block *et al* (*1*)	Kuroda	Kossel *et al* (*17*)	Kuroda	Kuroda
Iso-leucine	1.0	1.1		0.6	0.9
Valiee	3.6	1.5		1.6	1.2
Proline	8.2	3.9	3.8	3.7	1.1
Alanine	4.7	3.7	6.8	3.7	5.2
Threonine	1.9	1.8		0.9	1.0
Serine	3.4	1.8		1.8	5.2
Arginine	87.3 86.8	86.9	88.8	88.2	85.2

Clupein: of the sperma of *Clupea harengus*, Scombrine: of *Scomber scombrus*, Spheroidine: of *Spheriodes rubripes*.

DISCUSSION

Of the spot-dilution technique and its modification (*9*, *10*, *15*), we can expect only rough results. The ninhydrin test is very sensitive, but it is inconvenient because each amino acid gives its own particular colortone, so that a standard solution of each amino acid is needed to carry out accurate determination. Furthermore it is too labile to pH, temperature and reaction time.

The method described by Moore and Stein (*11*) seems excellent as a measurementmethod, but in spite of its complicated procedure and apparatus, it is not free from the defects of ninhydrin reaction.

The determination of copper in the complex with amino acids may give accurate values by polarograpyy (*12*). The method by the carbamate reagent (*13*) is far more simple and easy. Consequently, our modified copper method using carbon tetrachloride and hydroxylamine

instead of amylalcohol as the extractant (*14*) of copper diethyldithiocarbamate, has given more satisfactory results than any other previous methods for the analysis of amino acids in protamine.

SUMMARY

Amino acids of clupeine, scombrine and spheroidine were determined on their acid hydrolysates by the copper method combined with paper partition chromatography.

(A summary of this treatise was given as a lecture at the 22nd General Meeting of the Nippon Seikagakkai (Japanese Biochemical Society) on 27, April 1950. It was 3 March 1951, that a received and found a paper on a similiar subject by K. Felix *et al.* in *Z. physiol. Chem.* **286**, 67 (1950).)

REFERENCES

(*1*) Block, R. J., and Bolling, D., *Arch. Biochem.*, **6**, 417 (1945)
(*2*) Block, R. J., Bolling, D., Gershon, H., and Herbert, *A.*, *Proc. Soc. Exp. Biol. and Med.*, **70**, 494 (1949)
(*3*) Tristram, R. G., *Nature*, **160**, 637 (1947)
(*4*) Hamer, D., and Woodhouse, D. L., *Nature*, **163**, 689 (1949)
(*5*) Goto, M., *Z. physiol. Chem.*, **37**, 97 (1902)
(*6*) Hirohata, R., *J. Biochem.*, **10**, 251 (1929)
(*7*) Hirohata, R., *J. Biochem.*, **25**, 519 (1937)
(*8*) Kossel, A., *J. physiol. Chem.*, **25**, 165 (1898)
(*9*) Fisher, R. B., Parsons, D. S., and Morrison, G. A., *Nature*, **161**, 764 (1948)
(*10*) Polson, A., Mosley, V. M. and Wyckoff, R. W. G., *Science*, **105**, 603 (1947)
(*11*) Moore, S., and Stein, Wm. H., *J. Biol. Chem.*, **173**, 337 (1948)
(*12*) Martin, A. J. P., *Ann. New York Acad. Sci.*, **59**, 294 (1948)
(*13*) Woiwod, A. J., *Nature*, **161**, 169 (1948)
(*14*) Murakami, Y., *Bull. Chem. Soc.* (*Japan*), **23**, 3 (1950)
(*15*) Naftalin, L., *Nature*, **161**, 763 (1948)
(*16*) Pope, C. G., and Stevens, M. F., *Biochem. J.*, **33**, 1070 (1939)
(*17*) Kossel, A., (translated by Thorpe, W. V.), *The Protamines and Histones*, p. 33 (1928)

The Journal of Biochemistry, Vol. 38, No. 2.

CHEMICAL ASPECT UPON THE WATER COMPLIMENT AND THE SO-CALLED FOURTH COMPONENT OF THE COMPLEMENT

By MITSUNORI MARUYAMA

(*From the Biochemical Institute, Matsumoto Medical College, Matsumoto*)

(Received for publication, Feb. 10, 1951)

INTRODUCTION

In 1907 Sachs and Teruuchi (*1*) reported that when guinea pig serum was diluted with distilled water its complement activity for immune body disappeared. They diluted fresh guinea pig serum with nine volumes of distilled water and incubated at 37° for one hour, after-which sodium chloride was added to a concentration of 0.85 per cent. Thus treated serum showed no complement activity for sensitized corpuscles. They called the complement thus inactivated "water complement" and by this fact, they considered that the ferment-like substances in the serum would decompose the complement. The cause of water complement formation was investigated by Sachs and Altmann (*2*), Friedmann (*3*), and Leschly (*4*). Their explanations were stlll insufficient, since at that time toe components of the complement were not so clear as at present.

It was first found by Ferrata (*5*) that the complement consisted of endpiece and midpiece obtained by dialyzing the complement serum. Sachs and Omorokow (*6*), Omorokow (*7*), and Ritz (*8*) discovered the third component which was destroyed by cobura poison. Gordon *et al.* (*9*) also found the fourth component of the complement which was inactivated by ammonia. From studies on the water complement Kiyokawa (*10*) concluded that the cause of the formation of water complement might be due to the destruction of midpiece and subsequently, to the inability to combine with sensitized corpuscles. By adding heated serum or heated albumin fraction to the water complement, the activity was restored, and some other causes might be, therefore, expected.

According to Pillmer and his co-laboraters (*11*, *12*) the fourth component was inactivated by amine compounds, such as methylamine, ethylamine, or hydrazine. These compounds reacted with aldehyde where some aldehyde groups in the moiety of complement molecule were anticipated.

The author has been isolated a substance from the water complement, which inhibits the combination of midpiece with sensitized corpuscles, and it has been found that the substance is the fourth component which is reversibly denatured by water.

EXPERIMENTAL

Material and Method—As complement serum, guinea pig serum on was taken, an empty stomach and the sensitized corpuscles were prepared by treating 3 per cent ox corpuscles with 10 units haemolysin. The components of the complement were isolated as follows:

(a) Destruction of the third component: boil 8 per cent yeast solution in saline, add an equal volume of guinea pig serum, and incubate for 2 hours at 37° by stirring occasionally; remove the precipitate by centrifugation and dilute 10 times with saline.

(b) Destruction of the fourth component was performed by the method of Gordon, Whitehead and Wormall (*9*): Add 0.25 ml. of *N*/6.5 ammonia solution to 1.0 ml. of guinea pig serum, incubate for 1.25 hours at 37°, add 0.25 ml. of *N*/6.5 hydrochloric acid, and dilute 10 times with saline.

(c) Isolation of endpiece and midpiece was performed by Liefmann's method (1909) (*13*): Dilute 1.0 ml. of guinea pig serum with 8.5 ml. of distilled water and pass CO_2 stream through it for about 10 minutes; separate the precipitate from the supernatant by centrifugation, dissolve in 9.5 ml. of distilled water; add 0.5 ml. of 15.4 per cent saline solution to the supernatant and the dissolved precipitate. (The supernatant liquid is the albumin fraction, and the precipitate is the globulin fraction). Albumin and globumin fractions diluted 10 times were thus obtained. Before experiments it was confirmed that the fraction alone showed no complement activity, and that the complement activity was recovered by the combination.

The Demonstration of Water Complement and its Recovery—The water complement was prepared as follows: dilute 1.0 ml. of guinea pig serum with 8.5 ml. of distilled water, incubate at 37° for 30 or 60 minutes, and add 0.5 ml. of 15.4 per cent saline solution. In control test, phy-

siological saline solution was used instead of distilled water. In a series of test tubes, each 0.5 ml. of 3 per cent suspension of sensitized corpuscles was mix throughly with various amounts of guinea pig serum and kept at 37° for 1.5 hours, with which the degree of the hemolysis was examined. (Table I)

TABLE I

Amount of complement serum	Water complement						Control test					
	ml. 1.5	1.0	0.7	0.5	0.3	0.1	ml. 1.5	1.0	0.7	0.5	0.3	0.1
Time applying of water \ Degree of hemolysis												
15 minutes	±	±	−	−	−	−	##	##	##	##	##	##
30 minutes	−	−	−	−	−	−	##	##	##	##	##	##
60 minutes	−	−	−	−	−	−	##	##	##	##	##	##

As shown in Table I, when water was used, the complement activity was destroyed completely after 30 minutes. To obtain the water complement in the following experiments the water was applied for one hour.

The experiment were performed to determine whether the water complement could restore complement activity by adding the heated serum, albumin fraction or globulin fraction of guinea pig serum. The heated serum of guinea pig (at 56° for 10 minutes) was diluted 10 times with saline.

TABLE II

(a) *Water Complement (2.5 ml.)+Heated Serum (2.5 ml.)*

Amount of serum	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	−				−	−

(b) *Water Complement (2.5 ml.)+Alubumin Fraction (2.5 ml.)*

Amoun. of serum	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	−					−

(c) *Water Complement (2.5 ml.)+Golobulin Froction (2.5 ml.)*

Amount of serum	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	−	−	−	−	−	−

The water complement restored no complement activity by the addition of any fraction. It was shown by the experiments of (a) and (b) of Table II that the midpiece was destroyed in the water complement and that the another component of the complement was also inactivated (Table II, (c)).

Kiyokawa (*10*) reported that the water complement restored its action by heated serum or albumin fraction. But the inactivation of the complement by water was incomplete in his experiment and the water complement increased its action on the contrary by these frations.

Behavior of the Midpiece in the Water Complement—In the preceding section the midpiece was found to be altered in the water complement. The author has tried to perform to determine whether midpiece could be combined with sensitized corpuscles as in normal complement: add 5.0 ml. of 3 per cent sensitized corpuscles suspension to 5.0 ml. of the water complement, mix well, let stand for 30 minutes at room temperature, separate the corpuscles by centrifugation, wash 3 times with saline and then suspend it in 5.0 ml. of saline. As a control, the globulin fraction of guinea pig serum was used instead of water complement: add 2.5 ml. of the albumin fraction of guinea pig serum and 2.5 ml. of heated serum to each. The degree of hemolysis was observed after the incubation of one hour (Table III).

Table III

(a) *Water Complement+Sensitized Corpuscles+Alubumin Fraction (2.5 ml. +Heated Serum (2.5 ml.)*

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

(b) *Glublin Fraction+Sensitized Corpuscles+Alubulin Fraction (2.5 ml.)*

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	++++	++++	++++	++++	++++	++

The midpiece in the water complement could not be fixed with the sensitized corpuscles.

The Behavior of the Fourth Component, Endpiece and Third Component of the Complement—

(1) The fourth component—Dissolve sodium chloride in the water complement solution to a concentration of 0.85 per cent, let it stand for

30 minutes and then mix with an equal volume of guinea pig serum treated with ammonia solution. With this mixture the complement activity was tested (Table IV).

TABLE IV

Water Complement+Guinea Pig Serum Treated with NH_4OH.

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	-	-	-	-	-	-

From these results the fourth component is found to be inactivated in the water complement.

(2) Endpiece and the third component — With regard to the hemolysis of corpuscles by immune antibodies, and components of the complement were combined with corpuscles in the following order: midpiece, the fourth component, endpiece, and the third component. Therefore in the hemolysis test by using sensitized corpuscles, which had been previously combined only with midpiece and the fourth component,—if hemolysis occurred—with the water complement. Both third component and endpiece remained evidently active in the water complement. To obtain sensitized corpuscles combined with the fourth component, an equal amount of sensitized corpuscles which had been combined previously with midpiece and the heated guinea pig serum were mixed, left alone for 10 minutes at room temperature, and corpuscles were separated from liquid by centrifugation, and washed with the water complement or serum treated with ammonia solution. Hemolysis was observed after the incubation of 1 hour at 37° (Table V).

TABLE V

(a) *Sensitized Corpuscles which Combined with Midpieces and the Fourth Component+Water Complement*

Amount of suspension	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	++	++	+	+	±	−

(b) *Sensitized Corpuscles which Combined with Midpieces and the Fourth Component+Serum Treated with NH_4OH*

Amount of suspension	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	++	++	++	++	++	−

From these results it has been concluded that in the water complement the third component and midpiece are not inactivated completely.

Effect of Water and Temperature upon the Albumin and Globulin Fractions of Guinea Pig Serum—Guinea pig serum was fractionated by passing CO_2 into albumin and globulin, according to Liefmann (*13*) which were incubated for 1 hour at 37° in the abscence of sodium chloride. By these procedures the author obtained so-called "water albumin" and "water globulin." Using these fractions in the following combinations, the hemolysis tests were performed (Table VI).

TABLE VI

(a) *Water Alubumin (2.5 ml.)+Water Globul'n 2.5 ml.)*
(This mixture was incubated at 37° for 20 minutes and then NaCl was dissolved in a concentration of 0.85% and mixed with sensitized corpuscles.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	-	—	—	-	—	—

(b) *Normal Alubum'n (2.5 ml.)+Water Glubul'n (2.5 ml.)*
(The same treatment as above)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	+	+	+	+	±	—

(c) *Water Albumin (2.5 ml.)+Normal Glubul'n (2.5 ml.)*
(The same treatment as above)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

As shown in the result of (b) in Table VI, no essential change was observed in globulin fraction by the action of water; in other words, the midpieces remained free from chemical changes in the water complement. In the experiments of Table II and III, the midpiece was inactivated in the water complement. An explanation of this inconsistency will be given in a later section.

From the experiments of (c) in Table VI, the cause of the water complement formation seemed to be only a change of albumin fraction. The action of the water complement was not restored merely by the addition of albumin fraction since the change of the midpiece is definitive, it has been considered that the inhibition of hemolysis in experiment (c) is due not only to the change of water albumin fraction, but

also probably secondarily to the change of globulin fraction.

When sodium chloride was dissolved in the albumin fraction to a concentration of 0.85 per cent, and mixed with narmal globulin fraction after 30 minutes, hemolysis occurred as shown in Table VII.

TABLE VII

Water Albumin (2.5 ml.) Previously Treated with the Saline Solution +Globulin Fraction (2.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	+	±	—

The change in the water albumin was reversible and in the glbulin fraction some secondary changes were presumably induced by the altered albumin fraction.

Equal amounts of the water albumin fraction and the water globulin fraction were mixed, and incubated at 37° for 30 minutes; sodium chloride was dissolved to 0.85 per cent, and the behavior of the midpiece in this mixture were observed. For this purpose the same amounts of sensitized corpuscles were added to 5.0 ml. of this mixture. After the incubation for 10 minutes at 37°, the corpuscles were collected by centrifugation for the following hemolysis test (Table VIII).

TABLE VIII

Alubumin Fraction (2.5 ml.)+Heated Serum (2.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

The midpieces were inactivated in the presence of the both fractions. The fourth component was inactivated as already proved in Table IV. The inactivation of the water albumin fraction was reversible, and therefore, in the water albumin fraction, a substance altered reversibly has been considered to be the fourth cymponent altering irreversibly together with the midpiece.

The Effect of Water and Temperature upon the Alubumin and Globulin Fraction Separated by Ammonium Sulfate—The globulin fraction precipitated from guinea pig serum with ammonium sulfate at half saturation was washed twice with half saturated ammonium sulfate solution. The globulin fraction and the albumin fraction (supernatant) were dialyzed separately in collodion sack against running water. These fractions were

then treated with distilled water in the same manner as described above to obtain the water globulin and the water albumin. The experiments with these fractions were carried out as shown in Table IX.

TABLE IX

(a) *Water Albumin (2.5 ml.)+Water Globulin 2.5 ml.)*
(This mixture was left for 20 minutes at 37°, and then sodium chloride was dissolved to 0.85%. 0.5 ml. of sensitized corpuscle suspensions were added.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

(b) *Water Albumin (2.5 ml.)+Normal Globulin (2.5 ml.)*
(The same treatment as above)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	—	—

(c) *Normal Albumin (2.5 ml.)+Water Globulin (2.5 ml.)*
(The same treatment as above)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	–

The albumin fraction was not inactivated and the globulin fraction was inactivated irreversibly. The fourth component was removed in the globulin fraction when albumin and globulin fractions were isolated from guinea pig serum with ammonium sulfate (Table X).

TABLE X

(a) *Serum Treated with NH_4OH (2.5 ml.)+Globulin Fraction (2.5 ml.) +Sensitized Corpuscles*

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	+	—

(b) *Serum Treated with NH_4OH (2.5 ml.)+Albumin Fraction (2.5 ml.) +Sensitized Corpuscles*

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

Relation Between the Fourth Component and the Midpiece—Both the water albumin fractionated by use of ammonium sulfate and the water globulin

prepared by CO_2 treatment contained mostly no fourth component. Sensitized corpuscles were added to the mixture of these two fractions, and the midpiece were combined with them. The hemolysis test was carried out by using these sensitized corpuscles, the albumin fraction and heated guinea pig serum (Table XI).

Table XI

Alubumin Fraction (2.5 ml.)+Heated Serum (2.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	##	##

The results in Table XI showed that the midpiece was fixed with sensitized corpuscles, when the altered fourth component by water was not added.

The water globulin fractionated with ammonium sulfate contained the fourth component and the midpiece. The sensitized corpuscles were added to this fraction, and the experiment was performed to ascertain whether the midpiece would be fixed by corpuscles or not. For this purpose, normal albumin fraction and heated guinea pig serum were added to the corpuscles treated as above, and the degree of hemolysis was observed (Table XII).

Table XII

Alumumin Fraction (2.5 ml.)+Heated Serum (2.5 ml.)

Amouht of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

The fixation of midpiece by sensitized corpuscles was inhibited when the altered fourth component in the presence of water. The author shall call the fourth component inhibiting substance.

Adsorption of the Inhibiting Substance with Tonearth—The albumin fraction was isolated from guinea pig serum with CO_2 diluted 10 times with distilled water and incubated for 1 hour at 37°. 0.5 g. of tonearth was added to 8.0 ml. of the water albumin, and left to stand for 10 minutes at room temperature with occasional shakings. By centrifugation supernatant and precipitate were separated. The pH of the albumin fraction became about 4.5 when tonearth was added.

(a) Test of the inhibiting substance in the supernatant liquid—The supernatant liquid was neutralized and diluted twice with distilled water and left for 30 minutes at 37°. To this solution, 0.25 ml. of the water globulin was added and kept at 37° for 30 minutes, and then sodium chloride was dissolved to a concentration of 0.85 per cent. Sensitized corpuscles were added to it to examine whether the midpieces could be fixed or not. For this purpose, albumin fraction and heated guinea pig serum were added to the sensitized corpuscles treated as above and after an incubation for one hour at 37°, degree of hemolysis was observed. If hemolysis occurred, it would be evident that the midpiece is fixed with corpuscles.

TABLE XIII

Alubumin Fraction (2.5 ml.)+Heated Serum (2.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	+	+	+	+	+	—

As shown in Table XIII, there existed some inhibiting substance in the supernatant liquid.

(b) Test of the inhibiting substance in the precipitate—The precipitate was washed with distilled water until protein reaction disappeared, and suspended in 8.0 ml. of distilled water, adjusted to pH 8.0 and extracted for 10 minutes by stirring. The extract was separated by centrifugation and neutralized. The extract was mixed with the same amount of the water globulin and sodium chloride was dissolved in it to a concentration of 0.85 per cent and then the sensitized corpuscles were added to observe an occurrence of hemolysis.

TABLE XIV

Alubumin Fraction (2.5 ml.)+Heated Serum (2.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	+	±	±	±	±	—

The results of Table XIV showed that in the extract there existed the inhibiting substance; in other words, the inhibiting substance was adsorbed with tonearth by acid reaction and eluted by alkaline reaction.

As a control test, distilled water was used instead of the extract of

the precipitate and an experiment was performed in the same manner as above Table XV.

TABLE XV

Alubumin Fraction (2.5 ml.)+Heated Serum (2.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	+	—

No inhibiting reaction was observed in tonearth extract.

Relation Between Inhibiting Substance and the Fourth Component—In the previous experiments, it was clearified that the change of the water albumin was reversible. In this section an experiment was performed to decide whether the inhibiting substance would be reactivated by sodium chloride or not. The albumin fraction separated by CO_2 treatment contained the endpiece, the fourth and the third components. The distribution of these components in the supernatant liquid and in the extract of precipitate described in the previous section was examined. The supernatant liquid and the extract were used for experiment after dissolving sodium chloride in a concentration of 0.85 per cent and standing for 30 minutes. The sensitized corpuscles used in experiments for the proof of the endpiece had been previously combined with the midpiece (Table XVI and XVII).

TABLE XVI

Experiment with the Extract

(a) Proof of the endpiece:

Extract (4.0 ml.)+Heated Serum (0.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

(b) Proof of the fourth component:

Extract (4.0 ml.)+Serum Treated with NH_4OH (0.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	+	—	—

(c) Proof of the third component:

Extract (4.0 ml.)+Serum Treated with Yeast (0.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	±	—	—	—	—	—

Table XVII

Experiment with the Supernatant Liquid

(a) Proof of the endpiece:
Supernatant Liquid (4.0 ml.)+Heated Serum (0.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	##	—

(b) Proof of the fourth component:
Supernatant Liquid (4.0 ml.)+Serum Treated with NH_4OH (0.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	##	—

(c) Proof of the third component:
Supernatant Liquid (4.0 ml.)+Serum Treat with Yeast (0.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	+	—

As shown in these results of Tables XVI and XVII, the inhibiting substance in the water albumin fraction was eliminated partially when tonearth was added, and the inhibiting substance was found to be the fourth component altered temporarily by the water action.

Behavior of the Globulin Fraction Isolated from Water Complement—If the altered fourth component in the globulin fraction obtained by CO_2 treatment from the water complement was not interfered by an action of the midpiece, it must be fixed with sensitized corpuscles.

With sensitized corpuscles treated with the same amount of the water globulin fraction isolated from water complement the next experiment was performed (Table XVIII).

Table XVIII

Alubumin Fraction (2.5 ml.)+Heated Serum (2.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

As shown in the above result, in the water globulin fraction separated from water complement the inhibiting substance was combined with the midpiece and interfered by its fixation with sensitized corpuscles.

Effect of Proteinase upon the Fourth Component—Purified pancreatin was used as proteinase. The fourth component was prepared from guinea

pig serum by adsorption with tonearth and extracted by alkaline reaction as previously described. To 6.0 ml. of the extract, which had been examined to contain the fourth component, 2.0 ml. of 4 per cent solution of pancreatin was added and the reaction mixture was adjusted to pH 8.0 and incubated for 1 hour at 37°. For a control test, heated pancreatin solution was used.

TABLE XIX

(a) *The Fourth Component Digested with Pancreatin (4.0 ml.)+Serum Treated with NH_4OH (0.5 ml.)*

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	

(b) *The Fourth Component Designed with Heated Pancreatin (4.0 ml.) +Serum Treated with NH_4OH (0.5 ml.)*

Amount of mixture	1.5 cc.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	##	—

The fourth component was inactivated by pancreatin perfectly.

Chemical Properties of the Extract—With the extract obtained as above protein reactions and sugar reactions were tested, as shown in Table XX.

TABLE XX

(A) *Protein Reactions*

(a)	Colour reactions:	
	Biuret reaction	(+)
	Million's reaction	(+)
	Xanthoprotein reaction	(+)
	Leadrcetate reaction	(+)
(b)	Precipitation reactions:	
	Boiling with hydrochloric acid or acetic acid	(+)
	Sulfosalicylic acid	(+)
	Trichloracetic acid	(+)
	Almen's reaction	(+)

(B) *Sugar Reactions*

Nylander's reaction	(−)
Benedict's reaction	(−)

(Either by acid hydrolysis or by the action of heat, no sugar reactions appeared)

Effect of Hydrogenation upon the Fourth Component—Pillmer and his co-workers (*11*, *12*) reported that the substances easily combined with aldehyde, such as methylamine or ethylamine, inactivated the fourth component.

It can be considered that if aldehyde group is essential for the fourth component, its action must be destroyed by saturation of double bonds. For this purpose, the extract which contained the fourth component was hydrogenated. About 0.5 g. of platinum black was suspended in 20.0 ml. of the extract and through this mixture, hydrogen gas which had been purified successively by bubbling through the saturated mercuric chloride solution, 2 per cent permanganate solution and 5 per cent sodium hydroxide solution, was passed at 37° for 30 minutes, and the mixture was left for the sedimentation of the platinum black and then filtered. With this filtrate and guinea pig serum treated with ammonia solution, the hemolysis test was performed. As a control test, the extract which had been left with platinum black for 30 minutes without passing through dydrogen gas, was used in the same way.

Table XXI

(a) *Hydrogenated Extract (4.0 ml.)+Serum Treated with NH_4OH (0.5 ml.)*

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	—	—	—	—	—	—

(b) Control test:
Extract Treated with Paltinum Black only (0.4 ml.)+Serum Treated with NH_4OH (0.5 ml.)

Amount of mixture	1.5 ml.	1.0	0.7	0.5	0.3	0.1
Degree of hemolysis	##	##	##	##	—	—

The results indicated that the fourth component was inactivated by hydrogenation, and double bond was necessary for the action of the fourth component.

SUMMARY

1. When guinea pig serum was diluted with distilled water and left alone for 30 minutes at 37°, its complement activity disappeared.

2. The midpiece in the water complement could not be fixed with the sensitized corpuscles.

3. The fourth component existed in an inactive form in the water

complement.

4. The fourth component which had been freed from the midpiece was reversibly affected by the action of water and heat.

5. If the fraction containing both the fourth component and midpiece was treated with water at 37°, the latter turned to a state not to be fixed by sensitized corpuscles.

6. The substance selectively adsorbed by tonearth from the water albumin fraction, inhibited the midpiece to combine with sensitized corpuscles. This substance was converted to the fourth component by treating with sodium chloride. Therefore the substance inactivating the midpiece in the water complement was considered to be the fourth component altered by an action of water reversibly.

7. The midpiece in the globulin fraction separated from the water complement by CO_2 treatment was inactive, and, therefore, that midpiece was considered to be combined with the inhibiting substance in this fraction.

These facts may suggest that in the water complement the midpiece is combined with the altered fourth component and the former can not be fixed with sensitized corpuscles.

8. The fourth component was inactivated by digestion with pancreatin. The fraction containing only the fourth component showed the colour and the precipitation reactions of protein.

9. The fourth component was inactivated by hydrogenation. The double bond seemed, therefore, to be an essential structure of the fourth component.

The author wishes to express my sincerest thanks to Prof, S. Fujimura for his kind advices and encouragement in carrying out this investigation.

REFERENCES

(*1*) Sachs, H., and Teruuchi, X., *Berl. Klin Wschr.*, (1907)
(*2*) Sachs, H., and Altmann, K., *Handbuch d. Technik u. Methodik d. Immunitaetsforsch*, 2 (1909)
(*3*) Friedmann, U., *Z. Hyg.*, 67 (1910)
(*4*) Leschly, W., *Z. Immunitaetsforsch. Orig.*, 25 (1916)
(*5*) Ferrata, A., *Berl. Klin. Wsch.* Nr. 13. (1907)
(*6*) Sachs, H., and Omorokow, L., *Z. f. Immunitaetsforsch.*, Bd. 7 (1910)

(7) Omorokow, L., *Z. f. Immunitaetsforsch.* Bd. 10 (1911)
(8) Ritz, H., *Z. f. Immunitaetsforsch.*, Bd. 13 (1912)
(9) Gordon, J., Whitehead, H. R. u. Wormall, A., *Biochem. J.*, Vol. 20 (1926)
(10) Kiyokawa, N., *Kessei-gaku-meneki-gaku-zasshi*, Bd. 4, Nr. 2 (1943): Liefmann, H., *Muench. Med. Wschr.*, Nr. 41 (1909)
(11) Pillmer, L., Ecker, E. E., Oncley, J. L., and Cohn, E. J., *J. Exp. Med.* 74, 4 (1941)
(12) Ueno, M., *Tokyo-igakkai-zasshi*, Bd. 62, Nr. 6 (1936)
(13) Kobayashi, K., *Sansei-hakudo* (Maruzen) (1943)
(14) Nakagawa, Y., *Kessei-gaku-meneki-gaku-zasshi*, Bd. 1, Nr. 4 (1940)
(15) Pillmer, L., Seifter, J., and Ecker, E. E., *J. Immunol*, 40 2 (1941)

The Journal of Biochemistry, Vol. 38, No. 2.

STUDIES ON ω-OXIDATION. I. FATE OF L-RHODINIC ACID AND ITS ALLIED ACIDS IN THE ORGANISM

By RYOZO HIROHATA, SEIICHIRO YAMAZAKI, TOSHINORI ISEKI, TOKUWA TO, SYUN ISEDA AND MASATAKA SHOJIMA

(From the Departments of Medical Chemistry, Faculty of Medicine, Kyushu University, Fukuoka, the Department of Biochemistry, Affiliated College of Medicine, the former Taihoku Imperial University; the Department of Biochemistry, Faculty of Medicine, the former Taihoku Imp. University, and the Department of Chemistry, the former Institute of Tropical Medicine, Taihoku)

(Received for publication, March, 1, 1951)

INTRODUCTION

The destiny of L-rhodinic acid in rabbits has been already reported by Asano and Yamakawa (*1*). Nevertheless it seems worth while to note here the results of our own research. The probable existence of L-rhodinic acid in the essential oil of *Thujopsis dolabrata Sieb. et Zucc.* was reported by Kawamura (1930) (*2*) for the first time. Several years later, Nozoe (*3*) isolated it from the essential oil of *Chamaecyparis taiwanensis Masamune et S. Suzuki*, and later with the aid of his co-worker he ascertained that L-rhodinic acid is identical with L-2, 3-dihydrogeranic acid or the antipode of D-citronellic acid.

It was Shigehiro Katsura (*4*) who pointed out in 1941 the effect of rhodinic acid and its allied acids on lung tuberculosis. Since that time, one of us (R. Hirohata), as a co-worker of Katsura and Nozoe, has been investigating the change of the acids from an especial view-point, isolating intermediary products as possible effective factors. We administered the rhodinic acid orally not only to rabbits but also to a dog, cats and human beings, and obtained in each case, an ω-oxidation product, *i.e.*, dihydro-Hildebrandt acid from the urine. In the urine of rabbits to which relatively large amounts of L-rhodinic acid were ingested, we discovered more than 11% of the acid unchanged. Moreover, a small quantity of Hildebrandt acid, *i.e.*, a desaturated product of dihydro-Hildebrandt acid was isolated.

From the urine of human subjects to whom geranic acid had been given, Hildebrandt-, dihydro-Hildebrandt acid and unchanged geranic acid were obtained.

To cite an interesting instance, we isolated from the tetra-hydrogeranic acid-urine of a man Hildebrandt-, dihydro-Hildebrandt- and tetrahydro-Hildedrandt acids together with the given substance.

EXPERIMENTAL

A. L-Rhodinic acid

Material—L-Rhodinic acid which we used had the following properties:

$C_{10}H_{18}O_2$, b.p.$_{15}$: 149–51°, Sp.gr. $\frac{23}{4}$: 0.930–0.940
$[\alpha]_D^{20}$ (1 dm.): −5.5–−6.5° n_D^{20}: 1.4610–1.4615

Experiments on Rabbits—It was given to rabbits by mouth in a daily dose of 1 ml. per kilogram body weight as Na salt or free acid, to a total of about 400 ml. 20 rabbits were employed for 6 months. The urine of the rabbits was collected, acidified slightly with H_3PO_4 or H_2SO_4 and extracted with ether, and the ether was removed by evaporation. A crystalline substance mixed with the resinous matter was obtained. It was filtered by suction. The residue was recrystallized from hot water with norite. About 100 g. of colourless crystals of platelet were obtained, melted at 104°.

Analyses

Substance 4.093 mg.: CO_2 9.110 mg. and H_2O 2.997 mg.
Substance 4.373 mg.: CO_2 9.716 mg. and H_2O 3.200 mg.
Substance 4.840 mg.: CO_2 10.732 mg. and H_2O 3.507 mg.
$C_{10}H_{16}O_4$ (200.2) Calcd. C 60.00% H 8.06%
Found C 60.74, 60.63, 60.51% H 8.19; 8.19; 8.11%

Titration

Substance 6.017 mg.; *N*/100 NaOH, 6.52 ml.
Substance 6.761 mg.: *N*/100 NaOH, 7.30 ml.
$C_{10}H_{16}O_4$ Calcd. mol.wt.: 200.2
Found ml.wt.: 184.6, 185.2

bis-p-Bromphenacyl ester of dihydro-Hildebrandt acid melted at 121°.

Hydrogenation—Dihydro-Hildebrandt acid was hydrogenated with platinum oxide as a catalyst, and an oily substance was obtained, which turned crystalline after being stored a long time in the ice-box, m.p. 59-60°. R. Kuhn (*5*) described tetrahydro-Hildebrandt acid as an oily snbstance. Its *bis-p*-bromphenacyl ester melted at 109-10°.

The mother liquor of crude dihydro-Hildebrandt acid and the filtrate of its recrystallization were combined. From its ether solution, the acidic substances were taken in $NaHCO_3$ solution, freed in ether again by acdifying and dried with Na_2SO_4. About 350 ml. of a dark brown oily substance was obtained when the ether evaporated.

(a) 40 ml. of oily substance was converted into its ethyl ester and distilled under reduced pressure in 4 fractions. Each fraction was saponified and examined. Table I shows the results.

TABLE I

Fraction	Pressure mm.	Temperature	Yields ml.	Remarks
1	10	42°	5	0.8 g. crystal m.p.: 124°: benzoic acid
2	,,	86—8°	6	5.4 g. crystal m.p.: 124°: ,, ,,
3	,,	150—6°	20	14 ml. aromatic oil, with sharp taste, 26.0% COOH

(b) 100 ml. of oily substance was distilled as methyl ester with the results shown in Table II.

TABLE II

Fraction	Pressure mm.	Temp4rature	Yields ml.	Remarks
1	10	90°	13	13 g. benzoic acid, m.p. 124°
2	,,	150— 60°	45	(A)
3	,,	170—200°	24	(A)
Residue			16	

(c) 170 ml. of oily substance was also distilled as methyl ester: the results obtained are given in Table III.

(A) and (B) were combined and distilled as shown in the following Table IV.

TABLE III

Fraction	Pressure mm.	Temperature	Yields ml.	Remarks
1	10	80—100°	20	16 g. benzoic acid m.p. 124°
2	,,	140— 50°	60	
3	,,	160— 70°	23	(B)
4	,,	175— 90°	40	
Residue			19	

TABLE IV

Fraction	Pressure mm.	Temperature	Yields ml.	Remarks
a	10	89—90°	12	10 g. benzoic acid, m.p. 124°
b	,,	110—20°	15	12 g. ,, ,, 124°
c	,,	140—55°	72	
d	,,	160—75°	86	

Fraction c and d were saponified respectively and benzoic acid precipitated from the acidic part was discarded. The remainder was converted into its methyl ester again and distilled in vacuo as the following Table V.

TABLE V

Fraction	Fraction c				Fraction d			
	1	2	3	Residue	1	3	3	Residue
Pressure (mm.)	10	,,	,,		10	,,	,,	
Temperature	135°	145°	180–200°		135°	210–225°	240°	
Yields (ml.)	10	5	20	35	6	30	10	40
Remarks			I					
				II				
				III				

Collected fractions I: 21 ml., precipitated about 8 g. benzoic acid in a freezing mixture, m.p. 124°. The mother liquor 9 ml., was a yellowish pale aromatic oil and tasted sharp. It was distilled again and each fraction had the following properties (Table VI):

TABLE VI

Fr.	Press. mm.	Temperat.	Yields ml.	COOH %	Mol. weight.		m.p.	b.p.	Remarks
					Titrat.	m.-Rast			
1	3	85—94°	0.6				121		benzoic acid.
2	,,	100—30°	3.8	26.51	169.4	169.7		250	
3	,,	130—74°	3.1	45.91	196.0				
Residue			0.3						

Collected fractions II; 60 ml. Its acidic fraction, 42 ml., was esterificated with methanol and distilled as shown on the following Table VII:

TABLE VII

Fraction	Pressure mm.	Temperature	Yields ml.
5	3	85—110°	1.4
6	,,	110— 20°	3.2
7	,,	120— 30°	15.1
8	,,	130— 40°	16.6
9	,,	140— 50°	2.9
10	,,	150— 68°	2.7
Residue			0.2

Fraction 5 was saponified and a small amount of benzoic acid precipitated, m.p. 121°. From the fraction 6 was obtained some oily substance on saponifying and boiled at 250°.

Titration

$C_{10}H_{18}O_2$	Calcd. COOH 26.45%;	Found	26.51%
	Calcd. mol. wt. 170.2;	Found	169.4
Micro-Rast:	Calcd. mol. wt. 170.2;	Found	169.0

Collected fractions III, 75 ml., was treated as the above, and 42 ml. of methyl ester was obtained and distilled as follows (Table VIII):

TABLE VIII

Fraction	Pressure mm.	Temperature	Yields ml.
12	3	125—30°	2.8
13	,,	130—40°	8.4
14	,,	140—50°	4.3
15	,,	150—68°	7.8
Residue			0.2

The corresponding fractions of II and III were combined with each other and saponified. The results of examinations are tabulated as follows (Table IX):

TABLE IX

Fraction	Mol. weight		COOH %	b.p.	m.p.
	titration	m.-Rast			
12+ 7	172.7	171.5	26.32	253°	
13+ 8	173.7	172.1	26.16	255°	
14+ 9	197.5	198.2	45.50		193°
15+10	199.0	199.5	45.11		193°

The available evidence makes it very probable that fractions 2, 6, 7, 8, 12, and 13 are unchanged rhodinic acid. The total sum of the fractions was 49.9 ml. or 46.1 ml. free acid, that is, more than 11% of the ingested acid. Fractions 9, 10, 14, and 15; 17.7 ml. or 15.5 g. free acid in total, m.p. 190-2°, are probably Hildebrandt acid and showed no depression of melting point when mixed with pure Hildebrandt acid.

Experiment on Human Subjects—3 ml. of rhodinic acid was daily administered by mouth to 5 patients of lung tuberculosis till it totaled 237 ml. The collected urine was treated as above, and about 3.5 g. of dihydro-Hildebrandt acid was obtained together with benzoic and hippuric acids. From the mother liquor, the acidic substance was isolated as usual, converted into its methyl ester and distilled. The distillate

was sapoonified and the resultant acid was examined. The results obtained were as follows (Table X):

TABLE X

Fraction	Press. mm.	Temp.	Yields ml.	Mol. weight		COOH %	m.p.	b.p.
				titrat.	m.-Rast			
1	3	90—110°	2.0				120°	
2	3	110— 30°	4.0	171.7	171.4	26.25		255—60°
3	3	130— 56°	3.5	195.3	193.8	46.05		
Residue			0.2					

It is evident that fraction 1 is benzoic acid, fraction 2 rhodinic acid, and fraction 3 dihidro-Hildebrandt acid.

The fact that both unchanged rhodinic acid (1.5%) and dihydro-Hildebrandt acid (2.7%) were smaller in amount than those in the previous experiment will be probably due to the smaller dosage of rhodinic acid.

Experiment on the Dog and the Cat—The results were as follows (Table XI):

TABLE XI

Animal	Body wt. kg.	Dose, ml.		Urine ml.	Dihydro-Hild. acid. g.	m.p.
		daily	total			
dog	3.6	1	10	3750	1.3	102—3°
cat	1.26	0.5—0.6	10	2660	1.6	102°
,,	3.13	0.3—0.5	10			

This table shows that the ω-oxidation of rhodinic acid takes place in a dog and cats also.

(B) Geranic acid

Geranic acid was prepared by oxidizing citral with Ag_2O, which was obtained from linalool by treating with chromic acid mixture. It showed the following properties:

b.p.$_5$, 136—8°; sp. gr. $\frac{21}{4}$, 0.9571; n_D^{20}, 1.4896

52 ml. of the geranic acid in total was given orally to 6 patients of lung consumption for 2-9 days.

The collected urine was evaporated to syrup and extracted repeatedly with warmed 96% alcohol. The alcohol was evaporated from the extract and treated with ether. The residue of ether evaporation was kept in an ice-box. 3 g. of crystalline substance precipitated in several days and melted at 185°. The melting point rose to 192° after washing with benzene.

Substance, 3.560 mg.: CO_2, 7.920 mg.; H_2O, 2.20 mg.
$C_{17}H_{14}O_4$ (198.2) Calcd. C 60.60%; H 7.12%
Found C 60.71%; H 6.92%
Substance, 57.9 mg.: *N*/10 NaOH 5.80 ml.
$C_{10}H_{14}O_4$ Calcd. mol. wt., 198.2
Found mol. wt., 199.7

The mother liquor of it separated into 2 layers, the upper oily and the lower watery. Into the oily layer 65% H_2SO_4 (1/3 volume) was dropped slowly under cooling, and some crystals were produced to a quantity of 1.2 g., m.p. 104°.

Substance, 4.400 mg.: CO_2 11.460 mg., H_2O 3.580 mg.
$C_{10}H_{16}O_2$ (168.2) Calcd. C 71.40%; H 9.59%
Found C 71.08%; H 9.10%

It is very probable that geranic acid, excreted as such, changed to isogeranic acid through the above treatment, as Tiemann (*6*) claimed.

The watery layer was changed into an oily substance on being acidified with HCl. It turned into a crystalline mass in the ice-box. It was recrystallized from water and diluted alcohol, and there were obtained several fractions of crystals melting at 133°, 140°, 160°, and 172°, respectively.

Molecular weight of the fraction of m.p. 133°: 198
Molecular weight of the fraction of m.p. 140°: 197
Analysis of the crop of m.p. 140°
Substance 3.445 mg.: CO_2 7.680 mg., H_2O 2.200 mg.
For $C_{17}H_{14}O_4$ (198.2) Calcd. C 60.60%; H 7.12%
For $C_{10}H_{10}O_4$ (200.2) Calcd. C 59.99%; H 8.06%
Found C 60.84%; H 7.15%

It is probable that the substance is a mixture of Hildebrandt- and dihydro-Hildebrandt acid.

(C) Tetrahydrogeranic acid

The acid was prepared by oxidation of tetrahydrogeraniol, which

was obtained through hydrogenation of geraniol under pressure (180-200 kg./cm^2.) at 150-200° with Raney-Ni as a catalyst. It showed the following properties:

b.p.$_{12}$ 140--2°; sp.gr.$^{0°}_{4°}$, 0.8996; n^{20}_{D}, 1.4331

Non-existence of any double bonds was confirmed by the bromine test and an estimation of molecular refraction. It was given in a daily dose of 1 ml. orally to a patient of *tuberculosis pulmonum* for 58 days. The collected urine was treated as in the case of rhodinic acid and the acidic substance was distilled as methyl ester under reduced pressure (3 mm.) and examined as indicated in Table XII.

TABLE XII

Fr.	Temp.	Yields ml.	COOH %	Mol. weight		b.p.	m.p.	Remarks
				titrat.	m.-Rast			
1	70—100°	1.5					121°	benzoic acid
2	100—20°	2.6	26.65	168.5	169.4	250°		unchanged acid
3	120—30°	2.9	26.33	170.5	170.2	275°		unchanged acid
4	130—40°	6.9						broken while air transport
5	140—50°	2.7	45.22	199.0		302°	159--60°	dicarboxylic acid
6	150—55°	1.4	45.04	199.8				dicarboxylic acid
Residue		0.7						

As an available evidence shows, we may take fraction 1 as benzoic acid, fractions 2 and 3 as unchanged tetrahydrogeranic acid, fractions 5 and 6 as mixture of ω-oxidation products. Fraction 5 and 6 were combined together and recrystallized from water and the following 3 crops were obtained:

Crop a:

m.p. 188.5-90°, showed no depression of melting point when mixed with Hildebrandt acid, and its *bis-p*-bromphenacyl ester melted at 149.5-51.5°.

Substance 4.96 mg.: AgBr 3.22 mg.
$C_{26}H_{24}O_6Br_2$ (592.3) Calcd. Br 26.99%
Found Br 27.63%

Crop b:

m.p., 101-2°, melted at 99-102.5° mixed with dihydro-Hildebrandt

acid. Its *bis-p*-bromphenacyl ester melted at 120°.

Substance 6.14 mg.: AgBr 3.78 mg.
$C_{26}H_{26}O_6Br_2$ (594.3) Calcd. Br 26.89%
Found Br 26.20%

Crop c:

m.p., 49.5-57°. It showed no depression of melting point when mixed with tetrahydro-Hildebrandt acid.

$C_{10}H_{18}O_4$ Calcd. mol. wt., 202.2
Found mol. wt., 202.4 (titration)
Found mol. wt., 201.6 (micro-Rast)

Its *bis-p*-bromphenacyl ester melted at 109-10°.

DISCUSSION

More than 11% of L-rhodinic acid was put out unchanged in the urine of rabbits to which it had been administered in a relatively large quantity. The grade of ω-oxidation of undecylic acid in rabbits, which was at its highest hitherto described, did not exceed 5.8% (Verkade) (*7*). L-Rhodinic acid gave, on the contrary, more than 25% dicarboxylic acid, namely hihydro-Hildebrandt acid. This was probably due to the existence of methyl group at β-carbon of the acid, as Kuhn *et al.* (*5*) claimed. Another methyl group attached to the α-carbon of ω-carboxyl may also have prevented a further β-oxidation from ω-side as Lang *et al.* (*8*) and Flaschentrager and his co-workers (*9*) demonstrated. As a result of both causes it is increased in the yield of dicarboxylic acid.

According to Iijima (*10*) caprinic acid among the ordinal fatty acids shows the strongest antibiotic action to *Mycobacterium tuberculosis in vitro.*

On the other hand, we may conclude from the reports of Verkade (*7*) and Flaschentrager (*9*) that the ω-oxidation takes place to the largest extent at C_8-C_{11} fatty acids.

Thus, the optimum carbon atom number for ω-oxidation and antibiotic action on tubercle bacilli coincides approximately with each other. This coincidence could be considered to give some clew to clarifying the latter action. Contrarily to our expectation, however, the bactericidal action of dihydro-Hildebrandt acid on *M. tuberculosis in vitro* was not strong (private communication by Prof. S. Katsura).

Desaturation of fatty acid in the organism was deduced in and ter 1909 when the reports of Leathes (*11*) and Hartley (*12*) ap-

peared. In spite of many works such as those of Dakin (*13*), Sasaki (*14*), Schoenheimer and Rittenberg (*15*), Morehouse (*16*), and Carter *et al.* (*17*), we have had no definite proof of it, particularly of a fatty acid of natural sources.

Since the existence of citral geraniol or geranic acid in the essential oil of *Chamaecyparis taiwanensis Masamune et S. Suzuki* can be put out of consideration altogether, it is obvious that the origin of Hildebrandt acid in our case must be rhodinic acid or dihydro-Hildebrandt acid, or both of them.

Moreover the isolation of Hildebrandt- and dihydro-Hildebrandt acid from the tetrahydrogeranic acid-urine gives further conclusive evidence of the oxidation of fatty acids by desaturation at α, β-carbons. This desaturation may be regarded as an inductive phase of β-oxidation.

Elimination of such desaturation products is probably due to the difficulty of further β-oxidation owing to the branched chain at β-carbon on the one side and α-carbon on the other.

If such desaturation of fatty acid is taken into consideration together with hydrogenation, *e.g.*, formation of dihydro-Hildebrandt acid from Hildebrandt acid after the administration of geranic acid to man, the facts which were already regorted by Hildebrandt (*18*) and Kuhn (*5*), it is quite significant and needs further explanation.

SUMMARY

1. L-Rhodinic acid, when administered to a dog, cat, rabbits, and human beings, undergoes ω-oxidation to a large extent, and will be excreted in the urine as hihydro-Hildebrandt acid, the amount reaching about 25% of the ingested quantity in the case of rabbits.

2. A relatively large amount of unchanged acid will also be put out in the urine of rabbits and human beings, amounting in rabbits to more than 11% of the applied rhodinic acid.

3. Dihydro-Hildebrandt acid undergoes another oxidation, such as α, β-desaturation, and leaves the organism as Hildebrandt acid.

4. Hildebrandt- and dihydro-Hildebrandt acids were diminished in the urine together with tetrahydro-Hildebrandt acid after the application of tetrahydrogeranic acid to a man. It is a further evidence of α, β-desaturation of fatty acids in the organism.

5. Hildebrandt- and dihydro-Hildebrandt acids were isolated from the urine of a man to whom geranic acid had been administered.

6. Excretion of a large amount of ω-oxidation product of rhodinic

acid or allied substance, and of a detectable amount of desaturation product of di- or tetrahydro-Hildebrandt acid may be probably due to the hindrance of β-oxidation by the branched chain at β- or α-carbon, or both.

We are indebted to Prof. Katsura and Nozoe, and also to Takasago Chem. Industry Co. for research materials, and to Prof. Sebe for his valuable assistance in some experiments. This work was carried out partly by the aid of the Scientific Research Funds of the Ministry of Education.

REFERENCES

(*1*) Asano, M. and Yamakawa, T., *J. Biochem.*, **37**, 321 (1950)
(*2*) Kawamura, J., *Bull. Imp. Forest. Exp. Station* (Tokyo), **30**, 59 (1930)
(*3*) Nozoe, T., *Bull. Chem. Soc. Jap.*, **11**, 295 (1936)
(*4*) Katsura, Sh. *et al.*, *J. Med. Assoc. Formosa*, **40**, 1557 (1941); *Studia Med. Tropicalis*, Suppl. I, Taihoku (1944)
(*5*) Kuhn, R. *et al.*, *Z. physiol. Chem.* **220**, 235 (1933); **242**, 171 (1936)
(*6*) Tiemann, F., *et al.*, *Ber. deut. chem. Ges.*, **26**, 2726 (1893)
(*7*) Verkade, P. E. *et al.*, *Z. physiol. Chem.*, **217**, 225 (1933)
(*8*) Lang, K. *et al.*, *Z. physiol. Chem.* **263**, 227 (1940)
(*9*) Flaschenträger, B. *et al.*, *Z. physiol. Chem.*, **225**, 157 (1934); **238**, 221 (1936)
(*10*) Iijima, S., *Tohoku J. Exp. Med.*, **25**, 424 (1935)
(*11*) Leathes, J. B. *et al.*, *J. physiol.* **38**, xxxviii (1909)
(*12*) Hartley, P., *J. physiol.*, **36**, 17 (1907-8); **38**, 353 (1909)
(*13*) Dakin, H. D., *J. Biol. Chem.*, **6**, 203 (1909)
(*14*) Sasaki, T., *Biochem. Z.*, **25**, 272 (1910)
(*15*) Schoenheimer, R., *et al.*, *J. Biol. Chem.*, **113**, 505 (1936)
(*16*) Morehouse, M. G., *J. Biol., Chem.*, **129**, 769 (1939)
(*17*) Carter, H. E. *et al.*, *J. Biol., Chem.*, **128**, xiii (1939)
(*18*) Hildebrandt, H., *Arch., exp. Path. and Pharm.*, **45**, 110 (1901)
(*19*) Robinson, R., *J. Chem. Soc.*, **1904**, 505

The Journal of Biochemistry, Vol. 38, No. 2.

STUDIES ON LIPASE I. ON THE ACTIVATION OF PANCREAS LIPASE

By TOSHIICHI YAMAMOTO

(*From the Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Kyoto*)

(Received for publication, March 9, 1951)

Regarding the activation and inhibition of the lipase action, it must not be forgotten to take some chemical natures of the enzyme into consideration.

Glick *et al.* (*1*) have suggested from their precipitation tests that the chemical nature of the lipase concerns globulin, or it is closly related to globulin. It has been indicated by Murray (*2*) on the other hand that lipase contains an essential chemical group, which reacts with carbonyl of ketones. According to Weinstein and Wyne (*3*) the thiol compound plays a lipase stimulating action, while heavy metals, oxidizing reagents, ketones, aldehydes and iodoacetic acid *etc.* inhibit the lipase.

Bile acids are generally regarded as a lipase activator, on which are reported the observations by Kodama and Itoh (*4*), and the latter has especially demonstrated an activating effect of the reducing system as thiol and ascorbic acid and a depressing influence of the oxidizing system, which can also induce the fat synthesis.

It must be cited that Willstätter *et al.* (*5*) and Dawson (*6*) demonstrated an activating effect of polypeptide, such as L-leucyl diglycine and amino acids on the pancreas lipase, which according to Dawson seemed to be protected from an alkali idactivation in the presense of an amino acid.

This paper concerns the investigations of an accelerating effect of amino acids and some organic acids on the pancreatic lipase, prepared from pig pancreas by the acetone ether method.

EXPERIMENTALS

Lipase Assay with the Aid of Polyvinyl Alcohol as Dispersing Agents—Enzyme preparation: Pancreas powder was prepared according to Willstätter and Waldschmidt-Leitz (*7*) by the acetone ether method. This acetone powder obtained from fresh pig pancreas will maintain its activity more than one year when stored in a desicator, 1 g. acetone

powder of pig pancreas was ground and stirred with 200 ml. of 70% glycerol water at 37° for 3 hours.

The glycerol water extract was centrifuged for 10 minutes and the supernatant fluid was used as a lipase source. 4% gelatin solution, 1% polyvinyl alcohol solution, 4% sodium carboxy methyl cellulose solution, and 2% lauryl polyvinyl alcohol solution were examined in the test solutions for their dispersing effect. 5.6 g. olive oil was mixed with 160 ml. of each solvent and shaken vigorously for one hour, producing a fat emulsion of the final concentration of about $M/17$, taking olive oil as pure triolein.

Test solutions: It contains 3 ml. of 0.2 M NH_4Cl-NH_4OH buffer (pH 8.6)+2 ml. of 1% $CaCl_2$ solvtion+2 ml. of lipase solution+3 ml. of water+5 ml. of olive oil emulsion. Test solutions, adjusted to pH 8.6 with 1 N NaOH, were incubated under toluene at 37° for 4 and 24 hours.

At the end of digestion time, the acidity of 5 ml. of test solution was determined with the methyl alcoholic 0.1 N NaOH solution by means of a microburette using phenolphthalene as indicator.

Blanks without olive oil or without lipase were examined under the same conditions as the test solutions. Values of acidity of 5 ml. of main test solutions, corrected for these blanks, are given in Table I as "acidity increase" (ml. of 0.1 N NaOH).

TABLE I

Acidity increase in 5 ml. of digest (pH 8.6) (ml., 0.1 N NaOH)		
Dispersing agent of olive oil \ Time (hours)	4	24
1% Polyvinyl alcohol solution	0.56	0.74
4% Carboxy methyl cellulose-Na solution	0.53	0.72
2% Lauryl polyvinyl alcohol solution	0.53	0.72
4% Gelatin solution	0.57	0.76

Influence of Amino Acids on Pancreas Lipase (*pH* 8.6)—Enzyme preparation: The same preparation as in Table I. In the case of the triacetin test, there was used 2 ml. of a twice diluted enzyme solution.

Test solutions: 2 ml. of 0.2 M NH_4Cl-NH_4OH buffer (pH 8.6)+2 ml. of enzyme solution+1 ml. of 0.1 M amino acid solution adjusted to pH

8.6 with 1 *N* NaOH+0.17 g. of triacetin. Solutions for the olive oil test consist of 2 ml. of 0.2 *M* NH_4Cl-NH_4OH buffer (pH 8.6)+2 ml. of enzyme solution+2 ml. of 0.1 *M* amino acid solution (pH 8.6)+2 ml. of 1% $CaCl_2$ solution+5 ml. of olive oil emulsion in the 1% polyvinyl alcohol solution.

Control tests without amino acids were digested under the same conditions.

Blanks without substrates or without enzyme were examined.

Acidity increase in 1 ml. of the digest of triacetin test or 5 ml. of olive oil test is given in Table II. It is divided by the value of control, multiplied by 100 and shown in the table as the degree of hydrolysis, indicating a control value as 100.

TABLE II

Acidity increase in 1 or 5 ml. of digest (ml., 0.1 *N* NaOH)

Substrates / Time (hrs.) / Amino acids	Triacetin (*) (1 ml.)				Olive oil (†) (5 ml.)			
	1		24		4		24	
Control (No addition)	0.515	Degree 100	1.155	Degree 100	0.330	Degree 100	0.440	Degree 100
Glycine	0.546	106	1.282	111	0.390	121	0.519	118
L-Alanine	0.546	106	1.282	111	—	—	—	—
L-Leucine	0.551	107	1.293	112	—	—	—	—
L-Aspartic acid	0.762	148	1.894	164	—	—	—	—
L-Glutamic acid	0.675	131	1.640	142	0.416	126	0.580	132
DL-Lysine	0.772	150	1.813	157	0.505	153	0.612	139
L-Ornithine	0.783	152	1.825	158	0.554	168	0.708	161
L-Arginine	0.525	102	1.271	110	0.548	166	0.678	154
L-Histidine	0.804	156	1.930	167	0.740	224	1.082	246
L-Methionine	0.618	120	1.444	125	0.435	132	0.563	128
Urea	0.515	100	1.155	100	—	—	—	—
Taurine	0.556	108	1.259	109	—	—	—	—
Acetoamide	0.515	100	1.155	100	—	—	—	—

*) The final concentration of an added amino acid corresponds to about *M*/50.

†) The final concentration of an amino acid corresponds to about *M*/65.

Influence of Organic Acids on Pancreas Lipase (*pH* 8.6)—Enzyme and test solutions were similarly prepared as in Table II.

Only 2 ml. of 0.1 *M* organic acid solution (pH 8.6); was used instead of the amino acid solution. The final concentration of the acids is recorded in the table. The acidity increase, corrected for the blanks as in the previous tests, is given in Table III.

TABLE III

Acidity increase in 1 or 5 ml. of digest (ml., 0.1 *N* NaOH)

Substrates / Time (hrs.) / Organic acids	Triacetin (*) (1 ml.)				Olive oil (†) (5 ml.)			
	1		24		4		24	
Control (No addition)	0.525	Degree 100	1.140	Degree 100	0.340	Degree 100	0.440	Degree 100
Acetic acid	0.588	112	1.379	121	0.340	100	0.510	116
Lactic acid	0.540	103	1.230	108	0.305	90	0.410	93
Tartaric acid	0.530	101	1.277	112	0.296	87	0.395	89
Citric acid	0.704	134	1.652	145	0.420	124	0.590	134
Oxalic acid	0.483	92	1.185	104	0.285	84	0.374	85
Succinic acid	0.714	136	1.653	145	0.590	144	0.665	151
Fumaric acid	0.541	103	1.355	119	—	—	—	—
Maleic acid	0.708	135	1.573	138	—	—	—	—
Malonic acid	0.577	111	1.222	116	—	—	—	—
Pyruvic acid	0.430	82	0.957	84	—	—	—	—

*) The test solutions contain the approximately *M*/50 organic acid of final concentration.

†) They have about *M*/65 final concentration of the organic acid.

Influence of Derivatives of L-*Aspartic Acid and* L-*Glutamic Acid on Pancreas Lipase* (*Triacetin Hydrolysis at pH* 8.6)—All procedures are similarly carried out as in the previous tests (Tables II and III). 2 ml. of 0.1 *M* solutions of derivatives of both dicarboxylic amino acids were added to the test solutions, the final concentration being approximately 0.02 *M*.

TABLE IV

Acidity increase in 1 ml. of triacetin digest at pH 8.6 (ml., 0.1 *N* NaOH)

Derivatives of aspartic acid and glutamic acid (final concentration about 0.02 *M.*) \ Time (hours)	1		24	
Control (No addition)	0.482	Degree 100	1.130	Degree 100
Succinic acid	0.704	146	1.830	162
L-Aspartic acid	0.704	146	1.830	162
L-Glutamic acid	0.680	141	1.800	159
L-Benzoyl aspartic acid	0.709	147	1.829	162
L-Benzoyl glutamic acid	0.699	145	1.810	160
L-Asparagine	0.708	147	1.842	163

Influence of Lysine Derivatives on Pancreas Lipase (Olive Oil Hydrolysis at pH 8.6)—Enzyme and test solutions are prepared in the same manner as in the tests of Tables II and III.

TABLE V

Acidity increase in 5 ml. of olive oil digest at pH 8.6 (ml., 0.1 *N* NaOH)

Derivatives of lysine (final concentration about 1/65 *M.*) \ Time (hours)	4		24	
Control (No addition)	0.330	Degree 100	0.440	Degree 100
DL-Lysine	0.505	153	0.612	139
ε-Benzoyl-DL-lysine	0.415	126	0.490	111
α-ε-Dibenzoyl-DL-lysine	0.320	99	0.400	91

Amino Acid Effect on Alkali Inactivation of Pancrease Lipase (Triacetin Hydrolysis at pH 8.6)—Alkali treatment of enzyme solution: 2 ml. of original enzyme solution of pancreas powder used in the previous experiments, was diluted with 1 ml. of water, adjusting to pH 9.2 with 1 *N*

NaOH and incubated under toluene at 37° for 24 hours. This alkali treated enzyme-solution (3 ml.) was examined on its reduced activity of triacetin hydrolysis.

In the other cases to test the influence of added acids, 2 ml. of original eyzyme solution, mixed with 1 ml. of 0.1 *M* aspartic acid, succinic acid or asparagine solution, was incubated under toluene at 37° for 24 hours.

This enzyme solution was tested on the protecting action of the amino acid added. On the other hand, 2 ml. of original enzyme solution, adjusted to pH 9.2 with 1 *N* NaOH, was incubated at 37° for 24 hours and then mixed with 1 ml. of acid solution. This is used in the experiment to observe the reactivation of alkali-inhibited lipase by amino acids. After the treatment with alkali, each enzyme solution (pH 9.2) was adjasted to pH 8.6 with 1 *N* HCl.

Test solutions: 0.17 g. of triacetin+2 ml. of NH_4Cl-NH_4OH buffer (pH 8.6)+3 ml. of lipase solution, treated with alkali and mixed with acids to be examined. The test solutions were incubated under toluene at 37°.

Blanks without lipase or without triacetin were examined.

Control solution with 2 ml. fresh enzyme solution mixed with 1 ml. of water and 0.17 g. of triacetin in 2 ml. of buffer at pH 8.6. Values of acidity determidation corrected for blanks, are given as "acidity increase" in Table VI.

RESULTS AND DISCUSSIONS

As F. F. Nord *et al.* (*8*) have recently reported on the fat emulsifying action of polyvinyl alcohol, these synthetic compounds of high molecules as polyvinyl alcohol, lauryl polyvinyl alcohol and sodium carboxy methyl cellulose show about the same dispersing effect on the fat emulsion, such as gelatin, which is generally used as a fat dispersing agent in the enzyme study (Table I). Since gelatin is digestible by a crude lipase preparation, containing pancreatic protease, the polyvinyl alcohol is conveniently used in these experiments. The gelatin solution disturbs the end reaction in titration.

The observations demonstrate that dicarboxylic amino acids and diamino acids except arginine have a notable accelerating effect about to the 50% extent on the triacetin hydrolysis by pancreas lipase, while glycine, alanine, and leucine remain almost without influence (Table II). The olive oil hydrolysis was also in a similar degree stimulated by

TABLE VI

Acidity increase in 1 ml. of triacetin digest at pH 8.6 (ml., 0.1*N*NaOH)

Acids added (0.02 *M* in its final concentration) \ Time (hours)	1		24	
Control: Fresh enzyme without alkali treatment (No addition)	0.510	Degree 100	1.120	Degree 100
Incubation for 24 hours at pH 9.2 without addition	0.260	51	0.705	63
Incubation with aspartic acid for 24 hours at pH 9.2	0.440	86	0.983	88
After incubation for 24 hours at pH 9.2, aspartic acid is added	0.430	85	0.945	84
Incubation with asparagine for 24 hours at pH 9.2	0.383	75	0.963	86
After incubation for 24 hours at pH 9.2, asparagine is added	0.362	71	0.885	79
Incubation with succinic acid for 24 hours at pH 9.2	0.480	94	1.020	91
After incubation for 24 hours at pH 9.2, succinic acid is added	0.475	93	1.030	92

the addition of diamino acids, especially significantly by histidine, the hydrolysis value being twice as much as control. L-Methionine showed only an appropriate stimulation in both cases (Table II).

Among several organic acids examined, only the succinic and citric acid remarkably stimulated the pancreas lipase action on triacetin and olive oil (Table III).

It must be here pointed out that the maleic acid showed an accelerating effect on the triacetin hydrolysis, whilst fumaric acid remained almost without influence (Table III). This fact seems to suggest some relation of *cis-trans* isomerism of the compound to the lipase stimulation.

Table IV shows that benzoyl aspartic acid, benzoyl glutamic acid, and also asparagine exert almost the same accelerating influence upon the pancreas lipase action on triacetin as aspartic or glutamic acid.

The free amino groups of these dicarboxylic acids seem to be scarcely responsible for the lipase activation. On the other hand, one of both carbonyls appears to be without effect, since the compound retains the

stimulating influence when converted into acid-amide.

Regarding the effect of diammo acids, here is investigated the influence of lysine and its benzoyl derivatives on pancreatic lipase for olive oil hydrolysis. ϵ-Benzoyl-lysine indicates only a slight influence, whilst α-ϵ-dibenzoyl-lysine remains without effect on the lipase action.

These observations demonstrate the significance of both amino group of lysine.

Regarding the activating effect of aspartic acid and succinic acid on the pancreas lipase, the author has investigated the influence of these acids on the inactivation of the pancreas lipase by alkali (pH 9.2).

According to E. R. Dawson (1927) the activating effect of the amino acid, especially of the aspartic acid is due to its protecting action on the lipase from alkali inactivation. These observations indicate that the pancreas lipase, reduced to about 50% of its original activity, was reactivated almost to its original activity (85%) by the addition of aspartic acid, succinic acid or asparagine. The presence of these acids was not able to protect the pancreatic lipase completely from the alkali-inactivation but only in the same degree as reactivation by the acids.

It seems to suggest that the effect of these amino acids would be explained as reactivation of the pancreas lipase inhibited by alkali, rather than as protection.

SUMMARY

1. It is demonstrated that polyvinyl alcohol (1%), lauryl polyvinyl alcohol (2%) and sodium carboxy methyl cellulose (4%) are effective in emulsifying the natural fat about in the same degree as gelatin, and the polyvinyl alcohol is used as the most convenient dispersing agent in the experiments.

2. Dicarboxylic amino acids and diamino acids including ornithine have a notable stimulating effect (about 50%) on the pancreas lipase for the triacetin and olive oil hydrolysis, with the exception of arginine for the triacetin hydrolysis. An accelerating effect of histidine on the lipase for the olive oil hydrolysis is more than 100%.

3. Among several organic acids, succinic and citric acids are also effective (50%) on the lipase action. While fumaric acid remains almost without influence, maleic acid exhibits a notable stimulating effect on the lipase, as succinic acid. This fact suggests a certain significance of *cis*-form of these compounds.

4. With regard to the significance of the free amino group and the

carboxyl of the amino acids, benzoyl-L-aspartic acid, benzoyl-L-glutamic acid and L-asparagine show almost similarly an accelerating influence (50%) as free aspartic, glutamic or succinic acids. Therefore, the free amino groups of these two amino dicarboxylic acids as well as one carboxyl of aspartic acid seem to have little influence on the lipase.

Effect of lysine appears, on the contrary, to have its relation to both the free amino groups of the acid.

5. The pancreas lipase solution incubated with alkali at pH 9.2 and at 37° for 24 hours, loses 50% of its activity, which is not completely, but to about 85% protected from alkali inactivation in the presence of aspartic acid. When the acid is afterwards added to the alkali treated enzyme, it is also able to reactivate the loss of lipase activity to about 85%, as asparagine and succinic acid.

The author has been much indebted to Prof. Dr. Senji Utzino for his hearty guidance and helpful suggestions. These investigations owe much to the Ministry of Education for its grants for the Scientific Researches, for which the author wishes here to express his deep appreciation.

REFERENCES

(*1*) Glick, D. and King, C., *J. Am. Chem. Soc.*, **55**, 2445 (1933)
(*2*) Murray, D. R. P., *Biochem. J.*, **23**, 292 (1929)
(*3*) Weinstein, S. S., and Wyne, A. M., *J. Biol. Chem.*, **112**, 641 (1936)
(*4*) Itoh, R., *J. of Biochem.*, **27**, 279 (1938)
(*5*) Willstätter, R. and Memen, F., *Z. physiol. Chem.*, **138**, 216 (1924)
(6) Dawson, E. R., *Biochem. J.*, **21**, 398 (1927)
(*7*) Willstätter, R. and Waldschmidt-Leitz, E., *Z. physiol. Chem.*, **125**, 132 (1923)
(*8*) Fiore, J. V. and Nord, F. F., *Arch. Biochem.*, **23**, 473 (1949)

The Journal of Biochemistry, Vol. 38, No. 2.

STUDIES ON THE CONJUGATED LIPIDS. II. ON CEREBRON SULPHURIC ACID

By TEISHI NAKAYAMA

(*From the Department of Medical Chemistry, Faculty of Medicine, Hakkaido University, Sapporo*)

(Received for publication, March 12, 1951)

INTRODUCTION

Among the conjugated lipids in animal bodies, especially in the fraction of protagon, was found first by Thudicum (*1*) certain lipid containing sulphur which was called celled cerebrosulphatide by him.

Investigation on this lipid were later carried on by Koch (1910) (*2*), Levene (1912) (*3*) and Fränkel (1921) (*4*). These workers, however, seem to have dealt with impure materials which were not suitable for the study of the appropiate components and the codfiguration of this lipid.

In 1933 Blix (*5*) had taken up these problem, and devised a method of fractionation of sulphur containing lipids. He concluded that the sulphur containing lipids were composed of one molecule of cerebron and one molecule of sulphuric acid conjugated in ester linkage each other. He also concluded that the position of ester linkage would be at C_6 position of galactose in cerebron, as in case of other native sulphuric acid esters of sugar.

In previous publication (1950) the author (*6*) has reported on the configuration of cerebrosides, in which it was concluded that the glucosidic linkage in cerebron molecule should exist in the position of C_1 of sphingosine. And this time he wished to isolate this sulphur containing lipid and to determine the position of ester linkage between cerebron and sulphuric acid.

Since the yield of purified material was not sufficient enough (1.2 g.), definite method for the study of the configuration such as methylation of cerebron sulphuric acid could not be done.

The author, therefore, has selected following method for the present purpose: if the ester linkage of cerebron and sulphuric acid exists at the C_6 position of galactose as Blix claimed, no primary alcohol would be

found in the molecule of cerebron sulphuric acid. Making use of triphenylmethylchloride which is most effective reagent (*7*, *8*) for the identification of primary alcohol in the molecule of polyhydroxy compounds, experiments were carried out to see how this reagent reacts with cerebron sulphuric acid.

EXPERIMENTAL

Isolation of Cerebron Sulphuric Acid from Pig Brain—Crude protagon was separated by the method of Klenk (*9*) from 15 kg. of fresh pig brain, then treated with 85% boiling alcohol. Acout 280 g. of protagon (see first report (*6*)) were obtained.

Such protagon was treated in the same way as Blix carried out, *i.e.*, it was dissolved in five times volume of pyridine (b.p. 115°—116°) with occasional stirring at 60°, and the resulting precipitate was filtered off after cooling in an ice box for three hours.

The precipitate was dissolved in equal volume of pyridine once more, then cooled for one hour in an ice box. The precipitate was separated from mother liquid and washed thoroughly with acetone, then dried (90 g.). The residue was next dissolved in twenty times volumes of glacial acetic acid with stirring at 60°, after which it was cooled to 20° in water. The resulting precipitate was separated from mother liquid, treated again with one third volume of glacial acetic acid as before, and the residue was washed with acetone and dried (15 g.). The pale yellowish powdery one thus obtained was next treated with twenty times volume of chloroform-methanol mixture (3:1, vol.), cooled overnight in an ice box and filtered. The solution was then cooled for three hours at $-12°\sim-15°$ and the resulting large quantity of precipitate was collected by filtration and washing with the same mixture at the same temperature. After dryness it weighed about 9.5 g. The substance was again dissolved in twenty times volume of hot chloroform-methanol mixture (2:1, vol., about 180 ml.) and cooled for one hour at $-15°$.

The resulting precipitate was separated from mother liquid to get about 6 g. of white powdery amorphous crystal. Furthermore, it was recrystallized from chloroform-methanol mixture twice with 2:1 volume mixture followed by the same treatment with chloroform-methanol mixture (1:1, vol.).

Cerebron sulphuric acid thus obtained was snow white amorphous crystal, and showed the melting point of 209°—210°. It was insoluble

in ether, little soluble in alcohol and very easily soluble in hot chloroform (yield 1.2 g.).

Substance	49.60 mg.	N	Found	0.758 mg.
				1.53%
			Calcd.	1.48%
	81.20 mg.	S	Found	2.43 mg.
				3.42%
			Calcd.	3.38%

(Calculated as pottasium salt of cerebron sulphuric acid.)

Condensation Reaction between Cerebron Sulphuric Acid and Tri-Phenylmethylchloride—0.6 g. of cerebron sulphuric acid dissolved in 5.5 ml. of anhydrous pyridine was added to the solution of 0.18 g. of triphenylmethylchloride. The mixture was heated to some extent to get solution.

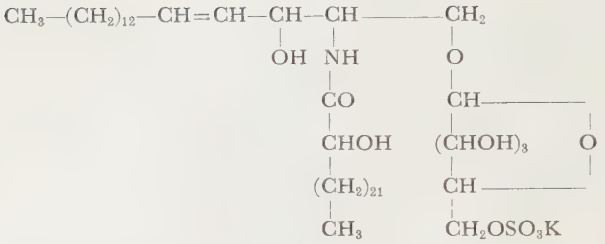
Under strict airtighting the mixture was left for 48 hours at room temperature, after which the solution was filtered. To the filtrate was added ice water at 0° until the content became turbid. The cooling was continued for half hour, then the solution was poured into 30 ml. of ice-water, the resulting white precipitate was filtered off which yielded about 0.15 g. This precipitate after three times of recrystallization from alcoholic solution showed the melting point of 159°. The mix-test with triphenylmethylalcohol (m.p. 159°) did not show any decrease of melting point. The qualitative analysis of nitrogen and sulphur gave negative result.

The mother liquid, when added to a small amount of alkali followed by evaporation of pyridine, showed cloudy precipitation again which could be extracted with chloroform. From chloroform layer, about 0.5 g. of unchanged cerebron sulphuric acid were recovered.

SUMMARY

The author isolated cerebron sulphuric acid after the method of Blix from pig brain.

To identify whether or not cerebron sulpuric acid has primary alcohol group, triphenylmethylchloride was made use of. The latter, however, did not react with the former, indicating any group of primary alcohol does not exist in the molecule of cerebron sulphuric acid. The configuration of cerebron sulphuric acid should be, as Blix reported, as follows:



The author wished to express his deep indebtedness to Prof. M. Yasuda for helpful critisism of this work.

REFERENCES

(*1*) Thudicum, J. L. W., *Diechemische Konstitution des Gehirns*, Tübingen, (1901)
(*2*) Koch, W., *Z. physiol. Chem.*, **70**, 94 (1910)
(*3*) Levene, E., *J. Biol. Chem.*, **13**, 463 (1912)
(*4*) Fränkel, S., and Gilbert, O., *Biochem. Z.*, **124**, 206 (1921)
(*5*) Blix, G., *Z. physiol. Chem.*, **219**, 82 (1933)
(*6*) Nakayama, T., *J. Biochem.*, **37**, 309 (1950)
(*7*) Helferich, B., *Ber. dtsch. chem. Ges.*, **158**, 872 (1925)
(*8*) Valentin, F., *Chem. Zentrabl.*, **103**, 1, 2160 (1932)
(*9*) Klenk, E., *Z. physiol. Chem.*, **267**, 168 (1940)

The Journal of Biochemistry, Vol. 38, No. 2.

MICRO-DETERMINTION OF RIBOFLAVIN, FLAVIN MONONUCLEOTIDE AND FLAVIN ADENINE DINUCLEOTIDE BY FILTER PAPER CHROMATOGRAPHY

By KUNIO YAGI

(*From the Department of Biochemistry, Nagoya Universtry School of Medicine, Nagoya*)

(Received for publication, March 20, 1951)

Since flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), derivatives of riboflavin, are coenzymes of important oxidases such as yellow enzymes, amino acid oxidase, xanthine oxidase etc., these are indispensable substances in biological oxidation. Besides, it has been also reported that free riboflavin itself has some significances on photochemical reaction in eyeball.

In 1933, Warburg and Christian (*1*) reported that they obtained lumiflavin as a derivative of flavin compounds by photolysis. Later Kuhn *et al.* (*2*) conducted various examinations of this procedure, by which they measured the quantity of flavin compound in tissues of animals and vegetables. Their method is as follows; extracting flavin compounds from tissues by methyl alcohol, adding NaOH to the extract, converting flavin compounds in the extract into lumiflavin by a two-hours' exposure to an electric lamp of 600 w., acidifying the extract with acetic acid, extracting lumiflavin from it three times with chloroform and measuring the colour of the chloroform layer by Pulfurich's photometer. It can be measured more accurately by its fluorescence than by its colour.

As a result of the various examinations conducted on lumiflavin fluorescence method (*3*), this has been proved to be an excellent one in determining flavin compounds of various tissues, especially of those containing many fluorescent substances.

With this method, however, only the total quantity of flavin compounds (calculated as free riboflavin) can be determined, because free riboflavin, FMN and FAD are all converted into lumiflavin. Accordingly, we cannot find out how much FMN or FAD is contained in the total quantity.

For the study of the physiological action of these three flavin com-

pounds *in vivo*, it is necessary to know the quantity of each of these three compounds in the tissues of the living body. For this purpose, the author tried to determine each of the flavin compounds by filter paper chromotography and the lumiflavin fluorescence method as mentioned above.

The method was based on the following principales: (1) The total quantity of flavin compounds (calculated as free riboflavin) is measured by the lumiflavin fluorescence method. (2) By filter paper chromatography, the three are separated from each other, extracted from filter paper and measured by the lumiflavin fluorescence method. From the result thus obtained the quantity of each flavin compound can be easily calculated.

As shown in experimental below, this method was considered applicable for the micro-determination of flavin compounds. As for filter paper chromatography the method reported by Crammer (*4*) was used. Here are reported the results of the examinations and practical details of this method.

EXPERIMENTAL AND RESULTS

According to the above mentioned principle, the method of the respective determination of flavin compounds in animal tissues is consisting of the following procedures:

(1) Extraction of flavin compounds from tissues.

(2) Determination of the total quantity of flavin compounds (calculated as free riboflavin) in the extract.

(3) Separation of the three flavin compounds in the extract by filter paper chromatography, that is, (i) removing protein from the extract with ammonium sulfate, (ii) extracting the flavin compounds with water-saturated phenol, (iii) returing them to water by the addition of ether to the phenol layer, (iv) placing the water solution on filter paper, (v) developing with a *n*-butyl alcohol acetic acid mixture.

(4) Separate extraction of each flavin compound from the filter paper and determination of each quantity (calculated as free riboflavin) by the lumiflavin fluorescence method to know the proportion of each one.

In adopting this procedure as a method for the respective determination of flavin compounds, the following conditions are required.

(1) In the operation for extracting flavin compounds from tissues, the compounds should be completely extracted in the solvent, during which time neither FAD nor FMN should be hydrolysed.

(2) In the operation for separating each flavin compound in tissues by filter paper chromatography, neither FAD nor FMN should be hydrolysed. Also in this procedure, the recovery of each flavin compound should be 100% or constant.

(3) In the operation for extracting flavin compounds from the filter paper, the three should be completely extracted or constantly recovered.

On the above items the following experiments were conducted.

Extraction Method—It is admitted that the methyl alcohol method of Kuhn *et al.* (*2*), the acetone method of Euler and Adler (*5*), the take-diastase method of Emmett *et al.* (*6*), and the method of heating in the presence of sulfuric acid of Hodson and Norris (*7*) are relatively good for extracting flavin compounds. The aim of all these methods is to extract flavin compounds in tissues as completely as possible, bu not to extract FMN and FAD without hydrolysis.

As all these methods are not satisfactory for the above mentioned requirement, the "warm-water-extraction" method was deviced (*8*). The procedure of "warm-water-extraction" is very simple and easy. The method is: (1) a piece of the tissue is excised, (2) it is cut immediately with a pair of scissors into the size of a red bean, (3) the small pieces of the tissue are immersed in water at 80° for 3—5 minutes, (4) they are ground by a homogenizer with that water and diluted with water to a degree suitable for determination, and (5) the diluted is solution heated at 80° for 15 minutes.

As my be seen by following results the method of "warm-water extraction" was proved to be the most suitable.

(1) When taka-diastase was placed in the extract of tissue, almost all the FAD and FMN were, as expected, changed into free riboflavin by nucleotidase and phosphatase contained in the taka-diastase.

(2) As a result of heating the flavin compounds in liver in the presence of sulfuric acid, it was found that a great amount of FAD was hydrolyzed. By this experiment it may be clear that the method of heating in the precence of sulfuric acid cannot be used for respective determination of flavin compounds,

(3) By the methods such as methyl alcohol extraction, acetone extraction and "warm-water-extraction", neither FAD nor FMN was hydrolysed.

(4) The total quantity of flavin compounds obtained by the acetone method was remarkably low, and in case of the other methods

the value was relatively high. But whenever the animal tissues were examined, the "warm-water-extraction" was the best for obtaining high values of the quantity of flavin compounds. Some of the results, for example, are shown in Table I.

TABLE I

The values are calculated as free riboflavin on the bassis of wet tissue (chrysalis of silk worm is dry) obtained by the lumiflavin fluorescence method with different extraction method (γ/g.).

Tissues \ Extraction method		Methyl alcohol	Taka-disatase	Heating in the presence of sulfuric acid	"Warm-water-extraction"
Chrysalis of silk worm		74.1	76.8	78.1	81.1
Frog	Skin (blak spot of the back)	108.0	120.0	116.0	122.0
	Liver	38.9	35.1	35.1	43.4
	Choroid	2120.0	2140.0	2170.0	2200.0
Guinea pig	Liver	30.0	30.0	30.0	34.9
	Kidney	29.1	32.1	32.1	32.1
	Muscle	1.3	1.6	0.8	2.9
Rabbit	Kidney	27.2	34.6	30.4	41.2
	Intestine	2.4	2.0	2.0	2.4

Filter Paper Chromatography—By use of FAD, FMN prepared from pig's liver (*9*) and riboflavin crystals synthesized by *Eremothecium* Ashybii, the following examinations were conducted:

(1) It was concluded that neither FAD nor FMN was hydrolyzed entirely throughout the procedure of filter paper chromatography, because in case of FAD or FMN only each of these appeared on the filter paper, and nothing else.

(2) The solution of each flavin compound was saturated with ammonium, sulfate, and aliquots were examined at 5th and 60th minutes after the saturation: neither decrease in the quantity of each flavin compound nor hydrolysis of FAD and FMN was observed.

(3) Each of the flavin compounds dissolved in 10 ml. of water saturated with ammonium sulfate was twice extracted with each 2 ml. of phenol, resulting the complete extraction of each flavin compound.

For dissolving a large amount of flavin compounds from the phenol

layer into water, ether had to be added twice or more as much as the volume of phenol layer.

From these facts, it was made clear that with this procedure neither FAD nor FMN were hydrolyzed and recoveries of these were almost 100%.

Extraction from Filter Paper—The paper was cut to pieces, immersed in water, and wamed at 80° for 15 minutes.

(1) FAD, FMN and riboflavin adsorbed on the filter paper were extracted to the same degree (about 90%) as far as the same filter paper was used. In the case of each flavin compound, moreover, similar recoveries were obtained with 4 different areas of the filter paper (2 cm. $\times$1 cm., 2 cm.$\times$2 cm., 2 cm.$\times$3 cm., and 2 cm.$\times$4 cm.), in which the same quantities were adsorbed respectively.

(2) Sometimes a minute quantity of phenol remained on the filter paper and when this coexisted with the flavin compounds after extraction, it greatly decreased the fluorescence of the latter. So it was an impediment for conducting the lumiflavin fluorescence method. However, when the filter paper was washed 2 or 3 times with ether, the phenol attached to it could be completely removed.

OPERATION

The following method of the respective determination of flavin compounds was adopted in accordance with the above mentioned results of the examinations.

Extraction("*Warm-Water-Extraction*")—A quantity (a g.) of the material to be determined is cut into the size of a red bean, placed in warm water at 80° for 3—5 minutes. Then the pieces of material are ground with that water by a homogenizer, diluted with water to an amount of b ml., warmed at 80° for 15 minutes, and after centrifugation the supernatant is used.

Measurement of the Total Quantity of Flavin Compounds (Calculated as Free Riboflavin)—

(1) Photolysis: 1.0 ml. of the extracted solution and the same volume of water are placed together in a glass-stoppered centrifuge tube (main test). Another 1.0 ml. of the extracted solution, 0.2 ml. of 200γ% riboflavin aqueous solution and 0.8 ml. of water are placed together in another tube (addition test). 2.0 ml. of *N*-NaOH are added to both tubes and they are illuminated by an electric lamp of 200 w. for 30—60

minutes at 20—40° solution temperature. Then 0.2 ml. of glacial acetic acid are added and after cooling at about 10° 4.0 ml. of $CHCl_3$ are added, the mixtures are shaken for 1 minute and 3.0 ml. of each of the $CHCl_3$ layer are transferred into non-fluorescent test tubes.

At the same time, 2.0 ml. of 200γ% riboflavin aqueous solution and 2.0 ml. of *N*-NaOH are placed together in another tube. Thereafter it is operated in the same way as the above two tubes, and 2.0 ml. of the $CHCl_3$ layer are transferred into a microburette (standard solution).

(2) Measurement: The fluorescence of the two tubes to be tested are examined by ultraviolet ray in a dark room. 3.0 ml. of $CHCl_3$ are placed in a non-fluorescent test tube, and titrated with standard solution until the intensity of fluorescence becomes the same as that in the tubes to be tested and the graduations of the microburette are read. Taking the graduation of the main test as f, that of the addition test as f', the quantity of flavin compounds (calculated as free riboflavin) contained in a material to be tested is $f \cdot \frac{0.3}{f'-f} \cdot \frac{4}{3} \cdot \frac{b}{a}$ γ/g.

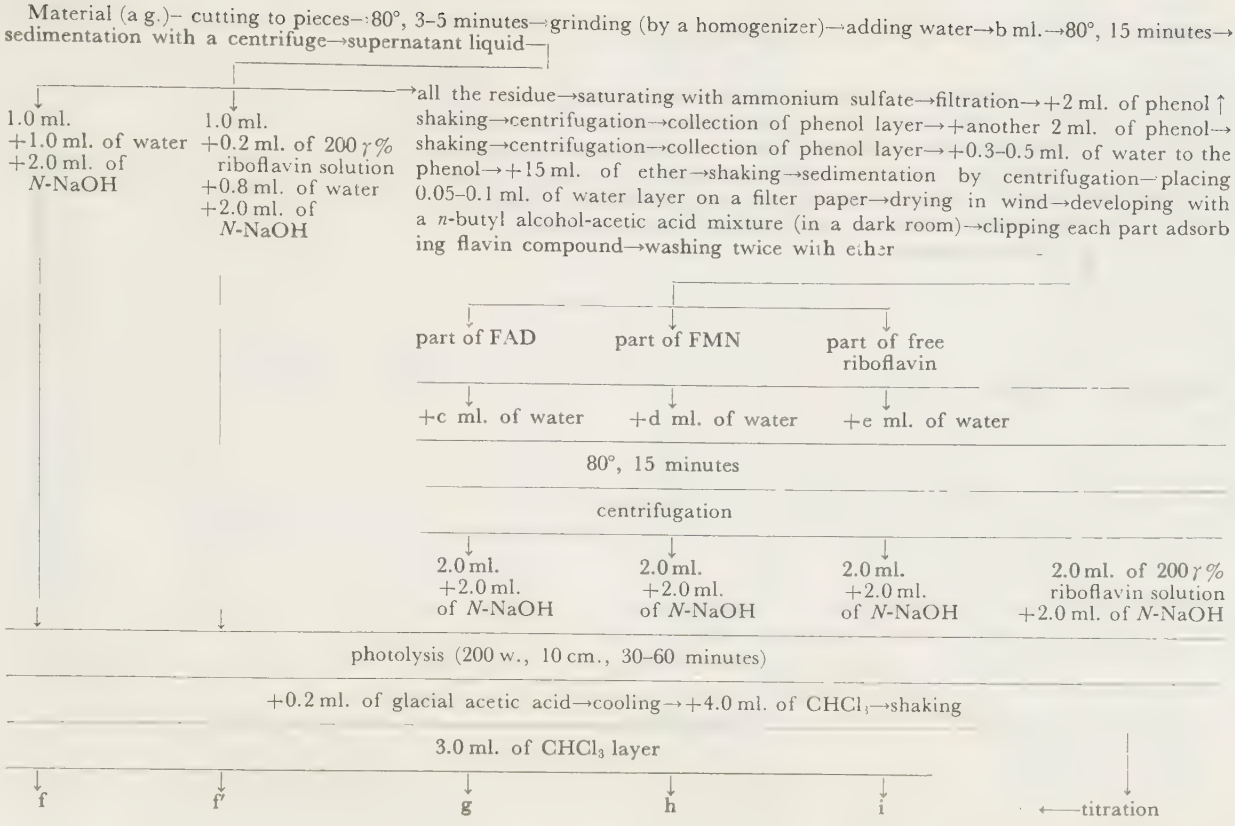
Ratio of Each Quantity of the Flavin Compounds—

(1) Filter paper chromatography (*4*, 10); All the residue of the extract is saturated with ammonium sulfate, and filtered. Then 2 ml. of water-saturated phenol are added to the filtrate, and after shaking the phenol layer is separated by centrifugation. The same procedure is repeated with another 2 ml. of water-saturated phenol. The phenol layer is collected in a glass-stoppered centrifuge tube, and 0.3—0.5 ml. of water and 15 ml. of ether are added. After shaking, the water layer is separated by centrifugation and 0.05—0.10 ml. of it are placed on a line about 5 cm. within the end of a filter paper (2 cm. $\times$30 cm.). After the filter paper is dried in the wind it is developed with a *n*-butyl-acetic acid mixture (4 parts of *n*-butyl alcohol is shaken with 1 part glacial acetic acid and 5 parts water, and the upper layer is used) in a dark room.

Each of the flavin compounds is examined by ultraviolet ray (Rf values of FAD, FMN, and free riboflavin are 0.02, 0.09, and 0.3, respectively), and the sections of paper adsorbing each of the flavin compounds are clipped separately from the filter paper.

(2) Reextraction: These sections of filter paper are washed with ether, cut to pices, and immersed in c ml., d ml., e ml., of water respectively. They are heated at 80° for 15 minutes and the supernatant is used for measurement.

Table II


Material (a g.)– cutting to pieces–80°, 3–5 minutes–grinding (by a homogenizer)–adding water–b ml.–80°, 15 minutes–sedimentation with a centrifuge–supernatant liquid–
all the residue–saturating with ammonium sulfate–filtration–+2 ml. of phenol–shaking–centrifugation–collection of phenol layer–+another 2 ml. of phenol–shaking–centrifugation–collection of phenol layer–+0.3–0.5 ml. of water to the phenol–+15 ml. of ether–shaking–sedimentation by centrifugation–placing 0.05–0.1 ml. of water layer on a filter paper–drying in wind–developing with a n-butyl alcohol-acetic acid mixture (in a dark room)–clipping each part adsorbing flavin compound–washing twice with ether
1.0 ml. +1.0 ml. of water +2.0 ml. of N-NaOH
1.0 ml. +0.2 ml. of 200γ% riboflavin solution +0.8 ml. of water +2.0 ml. of N-NaOH
part of FAD
part of FMN
part of free riboflavin
+c ml. of water
+d ml. of water
+e ml. of water
80°, 15 minutes
centrifugation
2.0 ml. +2.0 ml. of N-NaOH
2.0 ml. +2.0 ml. of N-NaOH
2.0 ml. +2.0 ml. of N-NaOH
2.0 ml. of 200γ% riboflavin solution +2.0 ml. of N-NaOH
photolysis (200 w., 10 cm., 30–60 minutes)
+0.2 ml. of glacial acetic acid–cooling–+4.0 ml. of CHCl3–shaking
3.0 ml. of CHCl3 layer
f
f'
g
h
i
titration

(3) Measurement: Each 2.0 ml. of c, d, and e are placed into glass-stoppered centrifuge tubes. They are operated in the same way as mentioned above. Taking g, h, and i as the respective graduations of c, d, and e, the ratio of each becomes cg: dh: ei.

All the above mentioned procedures are shown briefly in Table II.

Calculation—From the above mentioned results the quantities of FAD, FMN and free riboflavin (calculated as free riboflavin) are obtained by the following calculations.

$$\text{FAD}=f\cdot\frac{0.3}{f'-f}\cdot\frac{4}{3}\cdot\frac{b}{a}\cdot\frac{cg}{cg+dh+ei}\ \gamma/\text{g}.$$

$$\text{FMN}=f\cdot\frac{0.3}{f'-f}\cdot\frac{4}{3}\cdot\frac{b}{a}\cdot\frac{dh}{cg+dh+ei}\ \gamma/\text{g}.$$

$$\text{free ribobavin}=f\cdot\frac{0.3}{f'-f}\cdot\frac{4}{3}\cdot\frac{b}{a}\cdot\frac{ei}{cg+dh+ei}\ \gamma/\text{g}.$$

The absolute quantities of FAD and FMN, if necessary, can be easily calculated by multiplying the factors obtained from the molecular weights (FAD=above calculated value×2.09, FMN=above calculated value×1.21).

Examples of the Measurement—The quantities of flavin compounds contained in tissues determined by this method are shown in Table III.

TABLE III

The values are calculated as free riboflavin on the basis of wet tissue weight of white rats (γ/g.).

	FAD	FMN	Free riboflavin
Kidney	34.24	14.02	0.16
Liver	36.63	3.95	0.31
Heart	19.67	0.75	0.09
Stomach	6.78	0.78	0.14
Intestine	5.60	1.86	0.19
Spleen	6.05	0.54	0.13
Brain	2.74	0.61	0.06
Muscle	2.75	0.22	0.04

REFERENCES

(1) Warburg, O. and Caristian, W., *Biochem. Z.*, **266**, 377 (1933)
(2) Kuhn R., Wagner-Jauregg, T. and Kaltschmitt, H., *Ber., dtsch. chem. Ges.*, **67**, 1452 (1934)

(*3*) Yagi, K., *Modern Medicine* (*Gendai Igaku*) **1**, 9 (1950)
(*4*) Crammer, J. L., *Nature*, **191**, 349 (1948)
(*5*) v. Euler, H. and Adler, E., *Z. physiol. Chem.*, **223**, 105 (1934)
(*6*) Emmett, A. D., Bird, O. D., Brown, R. A., Pescock, G., and Vandenbelt, J.M., *Ind. Eng. Chem., Anal. Ed.*, **13**. 219 (1941)
(*7*) Hodson, A.Z. and Norris L.C., *J. Biol. Chem.*, **131**, 621 (1939)
(*8*) Yagi, K., Ishiguro, I., *Medicine and Biology* (*Igaku to Seibutsugaku*), **17**, 105 (1950)
(*9*) Yagi, K., *J. Jap. Biochem. Soc.* (*Seikagaku*), **22**, 162 (1950)
(*10*) Yagi, K., *f. Jap. Biochem. Soc.* (*Seikagaku*), **22**, 240 (1950)

The Journal of Biochemistry, Vol. 38, No. 2.

CHOLINE ESTERASE IN PIG SPERMATOZOA

By TAKAMITSU SEKINE

(*From the Department of Biochemistry, Faculty of Medicine, University of Tokyo, Tokyo*)

(Received for publication, March 22, 1951)

In 1921, Otto Loewi discovered that an active compound, the "Vagusstoff" is released if vagus is stimulated, and it was later identified with acetylcholine (Ach). Lowei's concept of "neurohumoral" transmission was widely accepted among physiologists. In 1933, Dale tried to extend this idea of a "chemical mediator" of the nerve impulse to the neuromuscular junction and to the ganglionic synapse.

Although his theory encountered strong oppositions, the problem of the role which Ach may have in the transmission of nerve impulses has led a great numbers of investigators to the studies on choline esterase (ChE), and "specific" ChE has been recently distinguished from "unspecified" ChE (*1*, *2*, *3*). Nachmansohn and his collaborators established a new chemical theory on the transmission of nerve impulses in connection with choline acetylase (*4*, *5*, *6*), namely, the "acetylcholine cycle" theory (*7*, *8*).

The vivid movement of pig spermatozoa by their tails suggests that they would represent an "elementary meuromuscular system," and would possess specific ChE, which has an important role in the acetylcholine cycle. This paper will present some fundamental researches on ChE in the spermatozoa, which should perhaps offer a new material for studies on mechanism of chemical processes in nerve and muscle.

METHODS

Spermatozoa—The pig spermatozoa used in these experiments were collected by means of artificial vagina (*9*, a) from normal ejaculates*, age of the animal ranging from one year and eight months to two and a half years. After the spermatozoa had been counted, the ejaculates kept at 15° were brought to our laboratory. The spermatozoa were

* The author wishes to express my heartly thanks to Dr. N. Yoshida and his collaborators of Zootechnical Experiment Station at Chiba for the supplies of these materials.

sedimented by centrifugation, washed twice with Ringer's solution, weighed and then resuspended to a concentration of 50 mg. per ml.

Brain Homogenate—Brains of mice, weighing about 15 g., were sliced and washed thoroughly with Ringer's solution to minimize the effect of contaminating blood ChE. The slices were homogenized and suspended in Ringer's solutoin.

Human Serum—Sera were collected from healthy men, and diluted with Ringer's solution to twenty-eightfold volume.

Substrates—Acetylcholine for clinical use were satisfactory for these experiments. Butyrylcholine chloride (Bch) was prepared in the following way: Choline chloride was heated with an excess of butyryl chloride in sealed tube at 100° for 3 to 6 hours. After the exsess of butyryl chloride was distilled off under reduced pressure, the reactive mixture was dissolved in water-free ethanol. The hygroscopic crystal of butyrylcholine was obtained by the addition of water-free ether.

An atempt to synthesize butyrylcholine perchlorate, which would be nonhygroscopic stable salt and exhibit pharmacologically the same effect as choloride (*10*), did not succeed. Benzoylcholine chloride (Bzch) was synthesized in analogous way as Bch. The crystalization of this compound was much easier than that of Bch. Benzoylcholine perchlorate (*11*) was not used in the experiments because of its little solubility.

Estimation of ChE Activity—The activity of choline esterase was determined manometrically by tee Warburg's technique at 37°. CO_2 liberated by acid formation from the choline ester from bicarbonate buffer was measured under a mixture of nitrogen and 5% CO_2. The activity values are expressed as:

$$Q_{Ach} = \frac{\text{mg. Ach hydrolyzed}}{\text{1 g. spermatozoa in wet weight/hour}}$$

$$Z_{Ach} = \frac{\text{mg. Ach hydrolyzed}}{\text{100 millions spermatozoa/hour}}$$

RESULTS

The motility—The motility of pig spermatozoa suspended in Cafree phosphate-Ringer's solution containing Mg (*12*) was activated by Ach and depressed by eserine. This fact suggested the significance of ChE in the movement of spermatozoa.

ChE Activity of spermatozoa—Table I shows the ChE activity of spermatozoa, ejaculated and epididymal, and of seminal plasma. It is

to be noticed that Q_{Ach} values of spermatozoa are comparable with those of rate, mouse, and ox brain observed by Nachmansohn (*3*). In the experiments presented here, however, much higher Q_{Ach} values for mouse brain were observed that in Nachmansohn's report. Henle and Zittle (*13*) observed that there was no great difference between rates of glycolisis of bovine epididymal and ejaculated spermatozoa. Also in ChE activity there was no great difference between the spermatozoa samples of both origins. Seminal plasma also has considerable ChE activity; one-third active as in human serum (Table II).

TABLE I

Choline Esterase Activity in Pig Spermatozoa and Seminal Plasma

(Spermatozoa 70 mg., seminal plasma 0.05–0.07 ml., final concentration of Ach 0.026 *M* in each vessel.)

Materials	Number	Weight of zoa mg. per ml.	Numbers of zoa 10^6 per ml.	CO_2 out put ml. per g. per hr.	Q_{Ach}	Z_{Ach}
Spermatozoa ejaculated	B22–38	23.4	3.27	2.57	18.7	0.134
	B22–38	20.1	2.38	1.56	11.4	0.096
	LY22–9	20.1	3.44	2.52	18.4	0.108
	LY22–10	35.8	3.48	1.99	14.5	0.149
	LY22–10	29.6	2.60	1.97	14.4	0.169
	B22–31	29.9	——	1.72	12.5	——
	Y22–13	27.1	3.42	2.01	14.7	0.117
	B22–1	29.5	3.83	2.61	19.1	0.147
Average		27.0	3.20	2.21	15.4	0.131
Spermatozoa epididymal	LY21–11	23.8*	——	2.46	18.0	——
Seminal plasma	Y22–60	CO_2 out put		148		
	LY22–9	μl. per 0.1 ml.		70		
	LY22–33	per hr.		107		
	B22–7			96		
Average				105		

* This weight is not absolute, because spermatozoa were washed out from epididymis by Ringer's solution.

Spermatozoa lost their ChE activity greatly in short time, unless the

temperature is kept at 15°; and no seasonal variation was found as their longevity (*9*, *b*).

From the average Z_{Ach} value of spermatozoa (0.131), one can calculate the number of Ach molecules, which will be splitted during one second by one spermatozoon. This turns out to be 1.35×10^6 molecules of the ester. Marnay (*26*) showed that in the motor end-plate of frog's sartorius muscle this is 1.6×10^9 molecules per millisecond per one end-plate.

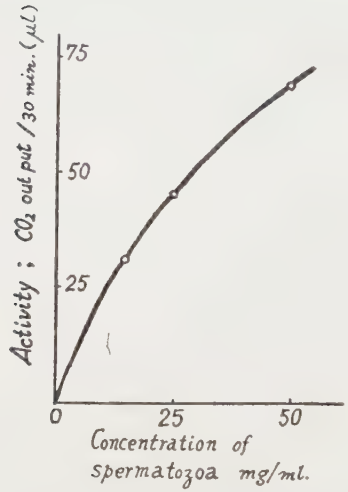


FIG. 1. Relation between Concentrations of Spermatozoa and Activity of Cholinesterase.

Final concentration of Ach is 0.026 *M*.

Relation between concentration of spermatozoa and ChE activity—

As shown in Fig. 1, the ChE activity tended to fall relatively, as the concentration of spermatozoa was increased. But such a maximum as found in respiration rate of epididymal spermatozoa (*13*) was not observed. In these experiments, the measurements were carried out in a concentration of approximately 50 mg./ml., because much higher concentration may cause some error due to protein effect.

Specificity of ChE of Spermatozoa—Two types of choline esterase have been distinguished by substrate specificities (*2*), relation between rate of hydrolysis and Ach concentration (*1*, *14*) or chloride effect (*1*, *15*). Nachmansohn and Rothenberg (*3*) emphasized the usefulness of comparing hydrolyzing capacities of enzyme applying different substrates. So, in these experiments, hydrolysing rates of acetylcholine, butyrylcholine benzoylcholine and tributyrine (Trb) by spermatozoa were compared with those by mouse brain (specific) and human serum (unspecified). The results are summerized in Table II. These data clearly shows that the ChE in pig spermatozoa is a "specific type," namely, it does not hydrolyze benzylcholine at all and splits less butrylcholine than Ach; yet Q_{Ach} of spermatozoa is distinctly higher than that of mouse brain.

The capacity of hydrolyzing Trb shows the presence of some unspecific esterase (*3*) and, it is quite curious, that this enzyme activity of spermatozoa is lost by homogenization for 10 minutes.

TABLE II

Specificity of Choline Esterase in Pig Spermatozoa

(Spermatozoa 70 mg., mouce brain 35–70 mg. and human serum 0.05 ml. in each vessel. Final concentration of substrates 0.026 *M*.)

Materials	CO_2 out put. μl per 0.1 g. or 0.1 ml. per hr. with Ach as substrate	Relative hydrolysability of different choline esters by choline esterase for Ach=100		
		Butyryl choline	Benzoyl choline	Tributyrine
Spermatozoa ijaculated	104	—	0	—
	162	69	0	37
	203	55	0	31
	145	—	0	—
	(155)	(22)	(0)	(0)
	(96)	—	(0)	(0)
Average	173	62	0	34
Mouce Brain	644	12	—	48
	866	21	2	53
	1660	12	1	46
Average	1057	15	2	49
Human serum	310	—	40	—
	257	176	—	—
	346	142	—	—
	188	163	—	90
	357	—	51	55
Average	292	160	46	73

Figures in brackets represent the values after homogenization, which are omitted in the calculation of average values.

Inhibitors—It is well known that two types of ChE are differently inhibited by certain inhibitors (*16*, *17*, *18*, *19*). Inhibitor experiments with caffein and quinine show again, as seen in Table III, that the ChE of spermatozoa belong to specific type. These observations about the ChE of brain and serum coincide well with those done by Nachmansohn (*18*).

TABLE III

Effects of Inhibitors on Choline Esterase in Spermatozoa, Mouce Brain, and Human Serum

(Concentration of inhibitors 2.5×10^{-3} *M*, Ach 0.026 *M*. Spermatozoa 60 mg., brain 30 mg. and serum 0.04 ml. in each vesel.)

Materials	Inhibition %							
	Caffeine		Chinine		Merzonine		Monoiodo-acetate	
Pig spermatozoa	(+5)	62	(0)	30	(0)	0	(0)	0
Pig seminal plasma		—		28		0		—
Mouce brain		29		26		0		0
Human serum		0		100		70		0

Figures in brackets represent motilities of spermatozoa observed instantly after the manometric estimations, contral being +5.

ChE is classified to SH-enzyme by Thompson (*20*). As toxic agents for SH group, monoiodoacetate and merzonine (ethyl meucuric thiosalicylate) (*21*) were tested for inhibitory action on ChE. While monoiodoacetate did not affect both types, merzonine showed strong inhibition of non-specific type. On the other hand, the enzyme in spermatozoa and braim was not inhibited by merzonine. Thus, merzonine can be used for eifferentiating both types ChE (*19*).

By the inhibitor experiment, it is suggested that ChE of seminal plasma belongs also to specific type.

DISCUSSION

The specific type of ChE which has been clarified to be unique in its restricted localization in bioelectrically active tissues, such as brain, nerve fiber, electric organ of electric fish and muscle, has now extended its existence to spermatozoa.

Bullock and Nachansohn (*22*) claim that the activity of ChE in the lower animals roughly pararells to the development of their nervous systems.

The activity of ChE in spermatozoa is as high as that in mammalian brains. If it is assumed that ChE distributes more abundantly in the tail, as infered from the function, the activity for unit weight in the tail is expected to be much higher. An experiment to disintegrate the

spermatozoa into heads, midpieces and tails by ultra-sonic vibration is now going on.

Here is noticeable that Q_{Ach} in spermatozoa is higher than that of the specific ChE in mammalian brain and ganglions, and is only comparable with giant fiber of squid, heart muscle of ox, optic never of *Raja* and whole body of *Planaria* (*3*, *23*). This fact suggests that the tails of spermatozoa resembles to the fibers mentioned in their mechanism of action.

While Ach, when applied on neuromuscular junction and ganglionic synapses, may develop a stimulating action, no action has yet been obtained when applied on axon. As Ach has conspicuous stimulating effect on motility of spermatozoa, it is assumed, therefore, either there be a region equivalent to neuromuscular junctions or synapses in spermatozoa (midpieces?), or their membran may be freely penetrated by Ach. Nachmansohn and his cooperators (*8*) firmly assert from a number of experimental evidences that such a substance, which is completely ionized and lipoid insoluble, does not penetrate the lipoid membrane of axon and can be expected to have no effect on nervous fibers. They suppose that the release and removal of Ach are an intercellular event and that Ach has a fundamental role in transmission not only at synaptic regions, but at axons (*8*, *23*, *24*, *25*). They comfirmed that inhibitors of ChE alter, or, in high concentration, abolish the action potential of nerve. If their hypothesis were right, these inhibitors would act in the same way on the movement of spermatozoa.

In fact, caffeine which inactivate the enzyme by two thirds does not affect the movement of the spermatozoa after incubation for 45 minutes (Table III). For the explanation of these conflicting events, further work is needed. One possibility is that one-third of the remaining activity may be enough for the function of spermatozoa.

Furthermore, several substances which have no inhibitory effect on ChE stifle completely the motion of spermatozoa. This leads to the conclusion that the whole process of the motion of spermatozoa involves so many steps that if even one of them is blocked by the inhibitor, the spermatozoa may loose their motility completely. Other observations indicate that there may be a mechanism regulating motion in which Ach may play a important role. Details will be presented elsewhere.

SUMMARY

1. The motion of pig spermatozoa is activated by Ach and depressed by eserine.

2. The average Q_{Ach} value of spermatozoa in ejaculate is 15.4 and that of epididymal spermatozoa 18.0. These values are comparable with that for ChE of mammalian brains. The number of Ach molecules split by one spermatozoon per second has been calculated from Z_{Ach} value of ejaculated spermatozoa to be 1.35×10^5.

3. ChE in pig seminal plasma is about one third active as in human serum.

4. ChE of spermatozoa does not splits benzoylcholine; is splits butyrylcholine in higher rate than the enzyme in brain does.

5. The enzyme in spermatozoa is inhibited by 62% with 0.0025 *M* caffeine, but not with monoiodoacetate. Merzonine inhibits the serum choline esterase by 70%, but has no effect on the activity of the ChE in spermatozoa or brain.

6. The motion of spermatozoa is stifled completely by quinine, merzonine and monoiodoacetate, but is not affected by caffeine which snhibits ChE in spermatozoa.

These facts indicate that pig spermatozoa has a high activsty of choline esterase, which is classified in "specific" type.

The author is most grateful to Prof. K. Kodama and Ass. Prof. H. Yoshikawa for their helpful advice; and Mr. T. Usui for his technical assistance.

This work has been aided by a grant from Ministry of Education and Pharmacological Research Foundation.

BIBLIOGRAPHY

(*1*) Alles, G.A., and Hawes, R.C., *J. Biol, Chem.*, **133**, 375 (1940)
(*2*) Mendel, B., Mundell, D.B., and Rudney, H., *Biochem. J.*, **37**, 473 (1943)
(*3*) Nachmansohn, D., and Rothenberg, M.A., *J. Biol. Chem.*, **158**, 653 (1945)
(*4*) Nachmansohn, D., and Machado, A.L., *J. Neurophysiol.*, **6**, 397 (1943)
(*5*) Nachmansohn, D., and John, H.M., *J. Biol. Chem.*, **158**, 157 (1945)
(*6*) Nachmansohn, D., and Weiss, M.S., *J. Biol., Chem.* **172**, 677 (1948)
(*7*) Nachmansohn, D., *J. Neurophysiol.*, **6**, 383 (1943)

(*8*) Nachmansohn, D., *Ann. New York Acad. Sci.* XLVII, Art 4. 395 (1946)

(*9, a*) Ito, S., Niwa, T., and Kudo, A., *Research Bulletin of Zootech. Exp. Stat.* (Minis. Agr. and Fores.), **55**, 1 (1948)

(*9, b*) Ito, S., Niwa, T., Kudo, A., and Mizuho, A., *Research Bull. Zootech. Exp. Stat.*, **55**, 17 (1948)

(*10*) Carr, C.J., and Bell, F.K., *J. Pharm. Exp. Therap.*, **91**, 169 (1947)

(*11*) Bell, F.K., and Carr, C.J., *J., Am. Pharm. Ass. Sci. Ed.*, **36**, 272 (1947)

(*12*) Lardy, H.A., and Phillips, P.H., *Am. J. Physiol.*, **138**, 741 (1943)

(*13*) Henle, G., and Zittle, C.A., *Am. J. Physiol.*, **136**, 70 (1942)

(*14*) Mendel, B., and Rudney, H., *Science*, **100**, 477 (1944)

(*15*) Mendel, B., and Rundney, H., *Science*, **102**, 616 (1945)

(*16*) Zeller, E.A., and Bisseger, A., *Helv. chim. Acta*, **26**, 1619 (1945)

(*17*) Harris, M.H., and Harris, R.S., *Proc. Soc. Exp. Biol. and Med.*, **56**, 223 (1944)

(*18*) Nachmansohn, D., *J. Biol. Chem.*, **1959**, 239 (1945)

(*19*) Adams, D.H., and Thompson, R.H. S., *Biochem. J.*, **42**, 170 (1948)

(*20*) Thompson, R.H. S., *J. Physiol.*, **105**, 370 (1947)

(*21*) Akiba, T., and Ishii, K., *Japan J. Bact.*, **5**, 23 (1950)

(*22*) Bullock, T.H., and Nachmansohn, D., *J. cell. and Comp. Physiol.*, **20**, 239 (1942)

(*23*) Bullock, T.H., Grundfest, H., Nachmansohn, D., and Rothenberg, M.A., *J. Neurophysiol.*, 10, 11 (1947)

(*24*) Bullock T.H., and Grundfest, H., *J. Neurophysiol.*, **9**, 253 (1946)

(*25*) Grundfest, H., Nachmansohn, D., and Rothenberg, M.A., *J. Neurophysiol.*, **10**, 155 (1947)

(*26*) Marnay, A., and Nachmansohn, D., *J. Physiol.* **92**, 37 (1938)

The Journal of Biochemistry, Vol. 38, No. 2.

IMPORTANCE OF CONTROLLING WATER CONTENT OF THE SOLVENTS FOR PAPER CHROMATOGRAPHY

By ATUHIRO SIBATANI and MICHIO FUKUDA

(*From the Research Institute for Microbial Diseases, Osaka University, Osaka*)

(Received for publication, April 5, 1951)

It has been pointed out recently by one of us, in collaboration with Takeda (*1*), that the water content of the developing solvents used in paper chromatography has a remarkable effect upon R_F values of amino acids in the case of such solvents as collidine-lutidine or butanol mixtures, which are saturated with water. This finding led us to a systematic investigation into the problem. As the use of various solvents completely miscible with water for paper chromatagraphy with addition of appropriate amounts of water had been suggested by Satake (*2*), experiments were extended with such solvents. Later, many papers published by English authors (*3*, *4*, *5*, *6*, *7*) came into our attention, which reported the use of water-miscible solvents and the controlling water content in them for paper chromatography of various substances to obtain R_F values satisfactory for experimental purpose. Also Stein and Moore (*8*) have announced the use of water-miscible solvents for starch column chromatography of amino acids. Application of simple distilled water to paper chromatography of inorganic compounds has also been suggested (*9*).

Under these circumstances, the classical theory of Consden, Gordon and Martin (*10*) of paper chromatography based on liquid-liquid distribution of the solutes must undoubtedly be replaced, or at least modified, by some others, for instance, such as proposed by Hanes and Isherwood (*5*) or Bentley and Whitehead (*4*).

The results reported here are, therefore, of confirmatory nature, for the most part, modifying the results reported by the preceeding investigators in several points of minor importance.

MATERIAL AND METHODS

The main part of the experiments was conducted with amino acids using the following solvents mixed with water on the graded levels of volume percentage: lutidine fraction, picoline fraction, pyridine, *iso-*

propanol, ethanol, methanol, acetone, acetic acid, *n*-butyric acid and phenol. Trinary mixtures such as *iso*propanol-ammonia, *iso*propanol-hydrochloric acid, methanol-chloroform and methanol-*p*-cresol, each being mixed with appropriate amounts of water, were also tested. Additional experiments on the paper chromatography of pyronin, a basic dye, and of some organic acids are mentioned briefly.

Lutidine fraction, picoline fraction and pyridine were purified through fractional distillation of commercial products, the boiling points of the first and the second being 140-160° and 120-140°, respectively. Phenol was distilled once with addition of zinc dust. As for other solvents, commercial preparations were used directly without process of purification. Amino acids were received from many laboratories.*

Tôyô Filter Paper No. 2 was used throughout the experiments. Solutions of amino acids were applified in 0.01 ml. portions on a line parallel to one side of the filter paper, drawn 3 cm. apart from the edge. 10-30γ of amino acids were placed on each spot, which is 2 cm. apart from the next. The paper sheet (30 cm.×30 cm.) was rolled into a cylinder, placed in a Petri dish containing the developing solvent, covered with a glass bell, and developed as the ascending chromatograph of William and Kirby (*11*). Saturation of the atmosphere with the developing solvent is certainly unsatisfactory during the first some ten minutes under such experimental conditions, but no serious errors may arise because of the ascending nature of the chromatograph. With phenol, mixed with water up to 40 per cent, small chromatograms (13 cm.×23 cm.) were developed in a desiccator which was placed in a 65° incubator.

The R_F values were measured according to Satake's extrapolation method (*2*): two or three spots were placed on the paper sheet serially, each containing the same amino acid decreasing in amount by the factor of $\frac{1}{2}$; after drying the paper at room temperature, the ninhydrin color was developed on a boiling water-bath, upper and lower ends of the serial ninhydrin spots obtained were connected with a pencil, and the two lines were extended to cross with each other. The cross point corresponds usually to the middle of each colored patch, but in some cases

* Thanks are due to Laboratories of Bacterial Chemistry Division and of Division of Bacteriology and Immunology, Research Institute for Microbial Diseases, Osaka University, of department of Chemistry, Osaka University and of Medico-Biological Institute, Minophagen Pharmaceutical Co., Tokyo, for the generous supply of many animo acid samples.

it approaches to upper or lower part of the spot. This method was necessary because the exact position of upper and lower ends is sometimes modified strongly by the amount of substance applied to the starting point.

It must be pointed out that most of the R_F values obtained by use of solvents with high water content may be too high, because the spot of each amino acids may travel some distance upwards during the first several minutes of drying at room temperature after removal of the paper from the developing chamber. (As the same time the solvent front moves also a little further.) Such "after-traveling" was first observed by Takanami, Kitazume, and Hirota (*12*) and confirmed by us in the case of distilled water as developing agent. Whereas the R_F values obtained through rapid drying of the paper by heating cluster around 0.90 in many amino acids developed with distilled water, those obtained after slow drying at room tempeaature reached altogether to values of as high as 0.99. The former values would reflect the displacement of substances more accurately, but drastic drying necessary for avoiding such "after-taaveling" appears unpractical with oaganic solvents because of the danger of firing, so that all the R_F values reported here were obtained without considering to minimize the after-traveling.

RESULTS

As already pointed out by Bentley and Whitehead (*4*), the R_F value of a given amino acid is increases with the amount of water containedin the organic solvents used. With simple distilled water as developing agent, all amino acids are scarcely retarded to the front of the moving phase. With pure organic solvents, on the other hand, amino acids give usually very low R_F values, moving not at all in many instances (Fig. 1). But with some solvents, such methanol (Table I) or *n*-butyric acid (Fig. 2), many of the amino acids investigated exhibit significantly high values. However, the spots obtained without addition of water show frequently a strong tendency to streaking, making the determination highly inaccurate, and in the case of 100 per cent phenol, the determination of the R_F's was completely impossible.

Between these extreme cases, namely, with mixtures of water and organic solvents, except for cases of very low water content, individual amino acids give usually discrete spots having characteristic R_F values which increase with water content of the solvents. With different

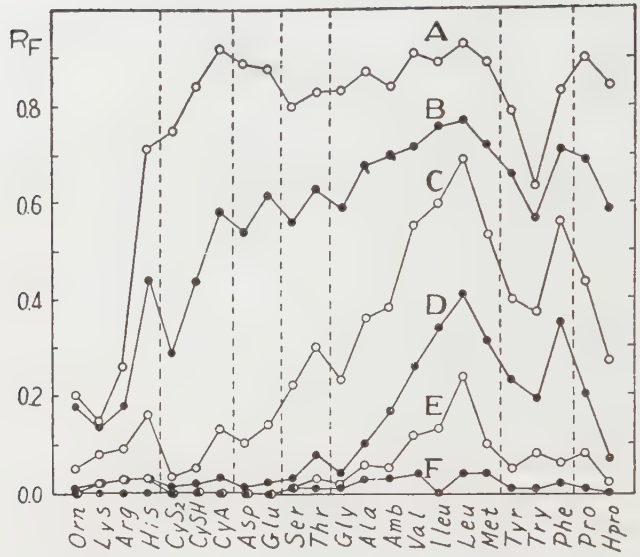


FIG. 1. Effect of water content (%, v/v) of *iso*probanol on the R_F values of amino acids. Water content (%, v/v): A, 70; B, 40; C, 20; D, 10; E, 5; F, 0.

Orn: ornithine, Lys: lysine, Arg: arginine, His: histidine, CyS_2: cystine, CySH: cysteine, CyA: cysteic acid, Asp: aspartic acid, Glu: glutamic acid, Ser: serine, Thr: threonine, Gly: glycine, Ala: alanine, Amb: α-aminobutyric acid, Val: valine, Ileu: *iso*leucine, Leu: leucine, Met: methionine, Tyr: tyrosine, Try: tryptophane, Phe: phenylalanine, Pro: proline, Hpro: hydroxyproline.

organic solvents, the distribution pattern of R_F values among the amino acids investigated are much alike, although some reasonable variations are always present. Usually long chain aliphatic acids and aromatic or heterocyclic acids give higher values than the others. Dibasic aliphatic acids are usually among the members with the lowest Rf values, but with *n*-butyric acid their R_F values are remarkably high.

It is worthwhile to point out that solvents belonging to the same group of organic compounds show remarkably similar patterns of the R_F's and that the increase of the molecular weight or of the boiling point of the solvents is reflected by the decrease of the R_F's when compared on the same level of water content. This fact was already

TABLE I

*Comparison of R_F Values Exhibited by Amino Acids Chromatographed with Pure Methanol, Methanol-Water, Methanol-p-Cresol-Water, and Phenol-Water**

Solvents	Methanol				Methanol-*p*-cresol-water (2 : 7 : 1)	Phenol	
	100%	90%	60%	30%		90%	60%
Ornithine	0.10	0.10	0.17	0.66	0.04	0.22	0.54
Lysine	0.05	0.07	0.13	0.16	0.13	0.17	0.55
Arginine	0.04	0.06	0.20	0.21	0.13	0.40	0.69
Histidine	0.08	0.12	0.59	0.76	0.24	0.64	0.75
Cystine	0.04	0.05	0.69	0.82	0.03	0.04	0.46
Cysteine	0.02	0.08	0.77	0.89	0.03	0.06	0.45
Cysteic acid	0.04	0.13	0.79	0.94	0.03	0.02	0.32
Aspartic acid	0.05	0.16	0.79	0.93	0.04	0.05	0.42
Glutamic acid	0.05	0.19	0.79	0.93	0.08	0.12	0.51
Serine	0.14	0.23	0.65	0.86	0.06	0.35	0.56
Threonine	0.34	0.46	0.70	0.90	0.14	0.49	0.64
Glycine	0.05	0.21	0.71	0.87	0.15	0.36	0.57
Alanine	0.40	0.50	0.80	0.90	0.43	0.63	0.73
α-Aminobutyric acid	0.44	0.56	0.78	0.90	0.46	0.82	0.86
valine	0.56	0.64	0.85	0.92	0.67	0.80	0.90
iso-Lueicine	0.57	0.67	0.80	0.91	0.69	——	0.96
Leucine	0.64	0.68	0.86	0.92	0.77	0.84	0.96
Methionine	0.35	0.50	0.85	0.91	0.62	0.83	0.88
Tyrosine	0.40	0.44	0.72	0.78	0.17	0.42	0.68
Tryptophane	0.36	0.38	0.54	0.60	0.55	0.71	0.90
Phenylalanine	0.42	0.57	0.76	0.86	0.76	0.86	0.96
Proline	0.32	0.43	0.82	0.91	0.76	0.90	0.98
Hydroxoproline	0.30	0.37	0.68	0.86	0.38	0.70	0.86

* Chromatography with phenol was conducted at 65°, otherwise at room temperature.

mentioned by Bentley and Whitehead (4) in the case of aliphatic alcohols, and we confirmed their results. Additional evidence was obtained by our experiments with pyridine derivatives (Fig. 3).

The main difference of R_F patterns between the above two groups of the organic solvents consists in the lower values of some amino acids

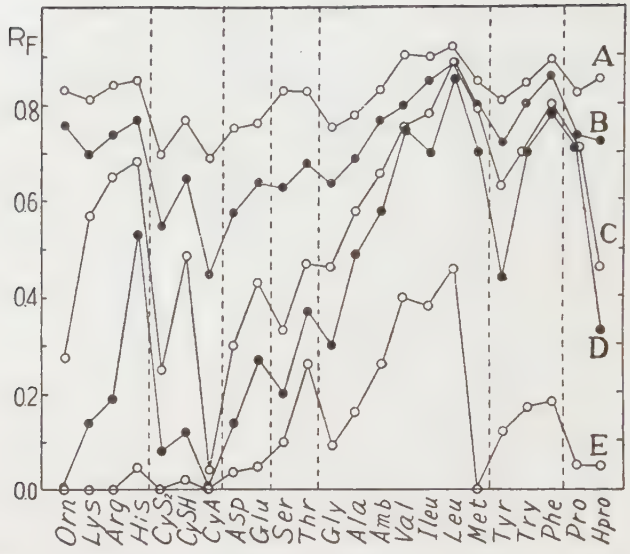


Fig. 2. Effect of water content of *n*-butyric acid on the R_F values of amino acids. Water content (%, v/v): A, 60; B, 40; C, 10; D, 5; E, 0. Abbreviations as Fig. 1.

such as aromatic group, especially tryptophane, cysteic acid and dibasic aliphatic amino acids with alcohols than with pyridine derivatives.

Acetone gives R_F pattern somewhat similar to the alcohols, but with this solvent the R_F values rise steeply with the increase of water content between 10 and 30 per cent in most of the amino acids, and it is in this region that the distribution of R_F values is favorable for practical purpose. Accordingly, the R_F values may fluctuate strongly even with the same water content because of the small difference of water content which is inevitably involved in the actual cases. With acetic acid the spots streak strongly even in the presence of significant amount of water admixed, so that the determination of R_F values has not been made. *n*-Butyric acid is characterized by high values of dibasic amino acids, cystine and cysteine—it is a remarkable fact that cystine and cysteine can be separated sharply by this solvent with moderate amount of water added—and low values of tyrosine and cysteic acid (Fig. 2). *n*-Butyric acid mixed with 5-20 per cent water will probably prove to be a good developing solvent of amino acids with very large range of R_F values

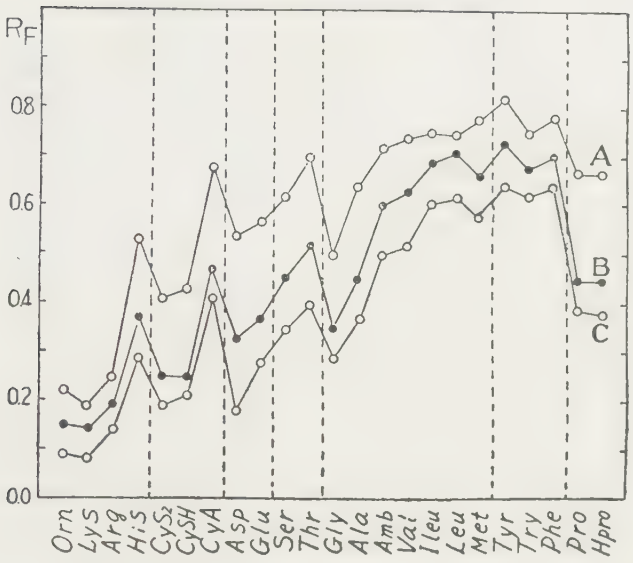


FIG. 3. Comparison of the R_F values of amino acids in pyridine derivatives containing 35% (v/v) water. A, Pyridine; B, Picoline; C, Lutidine. Abbreviations as FIG. 1.

(0.84–0.78) which is almost comparable to that of phenol.

Phenol exhibits a remarkable contrast against other solvents by the fact that the effect of the amount of water contained therein upon R_F values is far less significant than in the cases of other solvents (Table I). This is especially true for aliphatic monoamino-monocarboxylic acids. Dibasic amino acids give high and tyrosine relatively low R_F values.

Because the R_F values of amino acids are strongly modified by the water content of developing solvents, it will be important to search with any solvents an optimal water content for the separation of substances under investigation. For the most part of any pairs of substances, the magnitude of ratio of the two R_F values, or the "relative R_F" is higher with lower water content. In Fig. 4 changes of the relative R_F's of some amino acids to R_F's of alanine as a standard in relation to water content of *iso*propanol are given, the latter R_F's being converted to 0.5 (calculation: Relative R_F value to that of alanine as

$$0.5=\frac{R_F \text{ of a given amino acid}}{R_F \text{ of alanine}}\times 0.5).$$

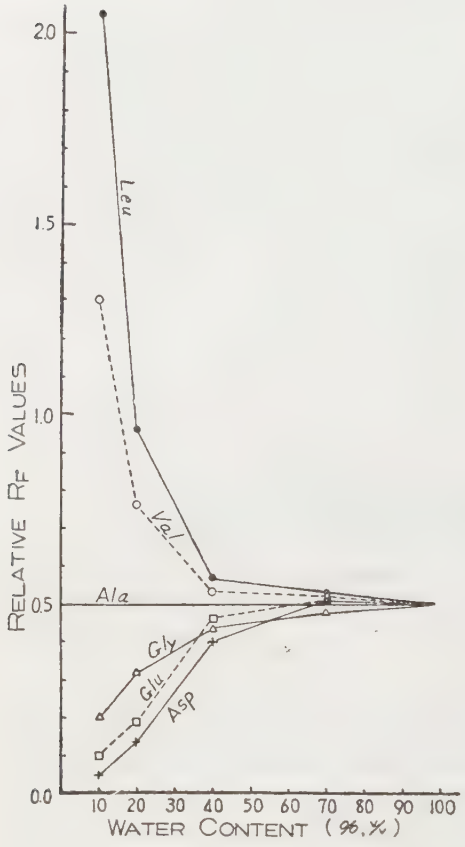


FIG. 4. Changes of the relative R_F values of some amino acids to R_F's of alanine as 0.5 in relation to the water content of *iso*propanol. Abbreviations as FIG. 1.

It should be pointed out that although the order of displacement of individual amino acids is usually not changed by the water content of the solvents, as already pointed out by Bentley and Whitehead (*4*), the existence of exceptions to this regularity was revealed in some instances in our experiments. In this respect the behavior of tryptophane was proved to be the most typical one. In Fig. 5 the R_F values for several amino acids chromatographed with ethanol are plotted against the water content of the solvent. Here, the curves of tryptophane and histidine and also those of phenylalanine and proline cross with each other at about 50 and 40 per cent of the water content, respectively. It is note-worthy that remarkably similar curves were obtained in two other alcohols tested, the corresponding crossing points showing reasonable shift towards higher water content in *iso*propanol (about 60 and 50 per cent) and lower water content in methanol (about 30 and 20 per cent).

Some variables of composition of the developing solvents other than water content were then investigated respecting their effect upon the R_F values. The effects of addition of acid and base were tested in the case of *iso*propanol containing 20 per cent water. Ammonia and hydrochloric acid appropriately diluted with water were added to *iso*propanol to make their final concentration 0.1, 0.3, 0.5, and 1 N and 20 per cent

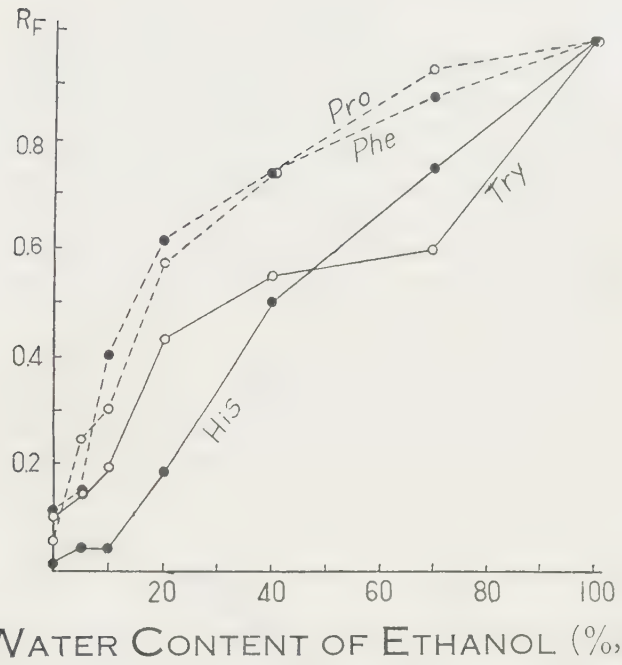


FIG. 5. R_F curves of some amino acid against the water content of ethanol. Abbreviations as FIG. 1.

in respect to water. The addition of ammonia was proved to shift R_F values of individual amino acids very little, if at all, from the values obtained with simple 80 per cent *iso*propanol. The addition of hydrochloric acid was, on the other hand, found to inhibit the color development of ninhydrin reaction, so that the estimation of the R_F values was impossible. The effect of addition of acetic acid was not investigated.

For the next, effect of introduction of hydrophobic compounds such as chloroform or *p*-cresol into a more hydrophilic solvent, methanol, was investigated. The replacement of methanol with different amounts of chloroform up to 70 per cent in the mixture of methanol and water, the volume percentage of the latter being maintained on the level of 10 per cent, gave R_F patterns of amino acids essentially similar to that of simple 90 per cent methanol. It is thus concluded that even high concentration of chloroform present in aqueous methanol exerts no appre-

ciable modification to the R_F values of amino acids obtained with the latter.

Effect of *p*-cresol replacing methanol in 90 per cent methanol upon R_F's of amino acid is somewhat more pronounced as compared with that of chloroform (Table I). Namely, the replacement of a part of methanol with *p*-cresol modified the R_F pattern in such a manner that some amino acids (phenylalanine, proline or leucines) give higher and some others (glycine, aspartic acid or serine) lower values. The over-all results become much approximated to the pattern shown by phenol. Therefore, it should be concluded that the difference of R_F values given by 90 per cent methanol from those given by methanol-*p*-cresol-water (2 : 7 : 1) is relatively small.

Thus, on the same level of water content, the replacement of very hydrophilic solvents such as methanol with more hydrophobic compound like *p*-cresol or chloroform brings about only minor or even insignificant effects upon the R_F values of various amino acids. This fact is in sharp

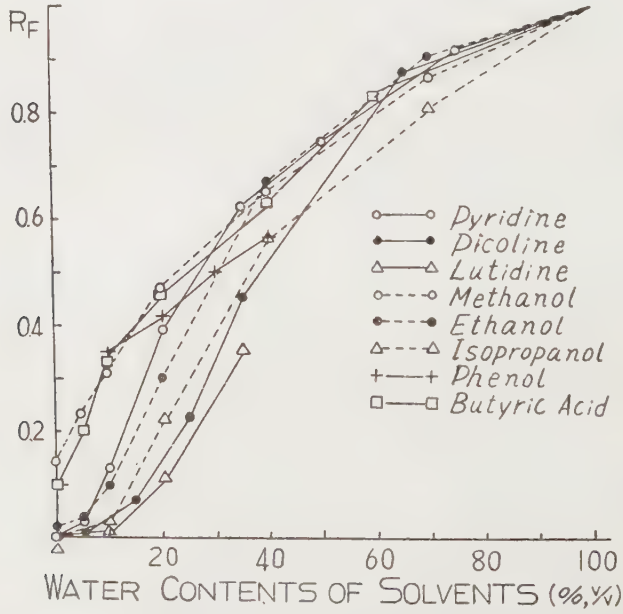


FIG. 6. R_F curves of serine obtained with various solvents against their water content.

contrast to the findings that the water content in most of the organic solvents introduces a considerable modification to the R_F values of amino acids. Indeed, the curves of the R_F values of most amino acids against the water content of varied organic solvents were found to be of more or less similar type as shown in Fig. 6 (serine); the remarkable separation of some of the individual cuaves as shown by lysine (Fig. 7) may rather be among the exceptional cases.

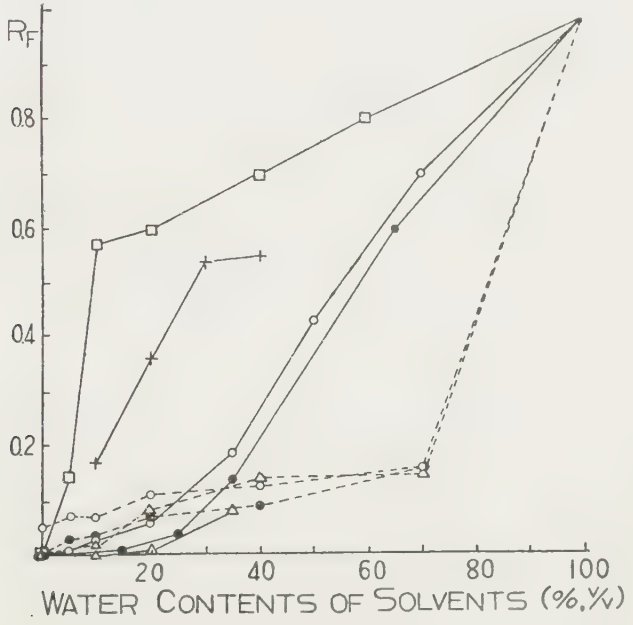


FIG. 7. R_F curves of lysine obtained with various solvents against their water Content. Individual solvents are distinguished as in FIG. 6.

DISCUSSION

We believe it has been well established in the above description of the results of our experiments that the water content of developing solvents for paper chromatography is among the most important as the modifying factors of the R_F values. In the case of amino acids, it may be fairly certain that no other substances employed as components of the chromatographic solvents exert such a general and considerable

effects upon the over-all distribution pattern of amino acids as water does. This does not, of course, rule out the possibility that for chromatographic separation of other groups of substances some components other than water have more significant effects upon the change of R_F values. However, it has already been confirmed that for chromatography of some other groups of organic compounds, the amount of water contained in the developing solvents is of importance of the same order as in amino acids. Isherwood and others (*5*, *13*) were among the first to devote their attention to the importance of water content in the developing solvent as a modifier of the R_F's of sugars and organic phosphoric esters. Markham and Smith (*6*) have shown a remarkable change of R_F values obtained with *n*-propanol-water in the case of adenine and adenylic acid. They have also pointed out that nucleotides travel a greater distance if solvents contain more water (*7*), although these authors as well as Magasanik *et al.* (*14*) have shown that the pH of the solvents is one of the most important factors affecting the R_F values of nucleotides. Also experiments conducted in this laboratory have proved that chromatographic behavior of basic dyes (pyronin and its relatives) and of aliphatic acids are modified considerably by the water content of developing agents.

Thus, it may be accepted that the water content of the developing agent for paper chromatography is usually the most important factor determining R_F values in a given solvent system, although some other factors such as pH may be sometimes of comparable importance. Therefore, it will always be highly desirable for obtaining good separation of compounds under investigation to control the water content of developing solvents. From the results reported above it may be expected that the best separation would be realized if the solvent front is allowed to run a very long distance and simultaneously the water content of the solvent is as small as possible within the limit not to cause the streaking of the spots, provided the substances to be chromatographed show significant, if slight, movement with such a solvent system. This poingt has already been emphasized by Hanes and Isherwood (*5*). However, it should be noted that for a group of substances the maximal R_F range or the difference of maximal and minimal R_F's among individual compounds of the group obtained with a given solvent system does not necessarily increase with the decrease of water content in the system employed. Instead, it can be seen from Table II that the maximal R_F range of amino acids investigated can usually be located at a certain

TABLE II

*Range of R_F Values Determined from the Over-All R_F Distribution of Amino Acids in Different Series of Solvent Systems of Varied Water Contents.**

Solvent system	Water content (volume per cent)													
	0	5	10	15	20	25	30	35	40	50	60	65	70	80
Phenol	—	—	0.88	—	0.87	—	0.78	—	0.66					
n-Butyric acid	0.46	0.84	0.85	—	0.78	—	—	—	0.44	—	0.23			
*iso*Propanol**	0.04	0.15	0.36	—	0.74	—	0.65	—	0.22	—	—	—	—	0.26
Ethanol**	0.04	0.24	0.40	—	0.66	—	—	—	0.48	—	—	—	0.30	—
Methanol**	0.16	0.38	0.53	—	0.65	—			0.36	—	—	—	0.33	—
Pyridine	0.62	0.63	0.56	—	0.43	—			0.32	—		—	0.34	—
Picoline fraction	0.05	0.29	0.54	—	0.69	—	—	0.60	—	0.51	—	—	0.23	—
Lutidine fraction	0.00	0.09	—	0.43		0.58	—	0.59	—	—	—	0.43	—	—
	0.00	—	0.20	—	0.53	—	—	0.56						

* The differences of maximal and minimal R_F's are presented irrespectively of species of amino acids which give these values.

** Dibasic amino acids are excluded because their R_F's are considerably low than those of the remainder in high water contents.

water content more or less higer than the lowest water content, the application of which affords clear-cut ninhydrin spots without streaking. This point is somewhat different from the conclusion given by Bentley and Whitehead (*4*). The same holds also for most amino acid pairs: as shown in Fig. 8, the peaks of the difference of R_F values of any two amino acids are sometimes situated at some moderate water content.

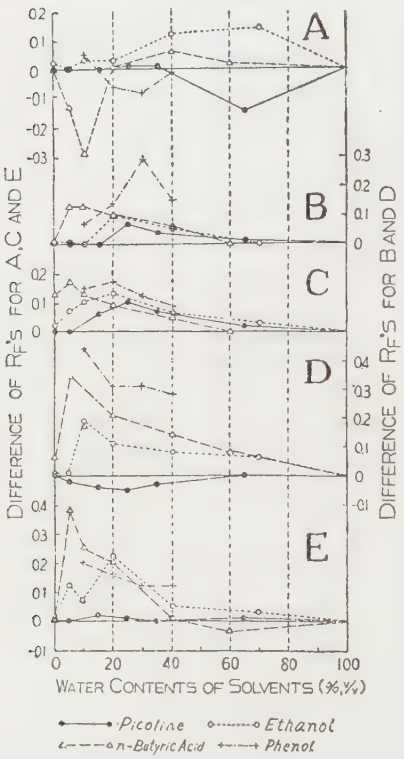


Fig. 8. Change of the difference of the R_F values of some amina acid acid pairs in relation to the water content of various solvents. A, Lysine-ornithine; B, Aspartic acid-glutamic acid; C, Serine-threonine; D, Tyrosine-phenylalanine; E, Proline-hydroxyproline.

If one applies the method of descending paper chromatography originally described by Consden, Gordon and Martin (*10*) or the bridge unit recommended by Kawearu (*15*), the solvent can be allowed to flow off the end of filter paper. Under such conditions resolving capacity satisfactory for separating compounds with approximate R_F values may be obtained even with solvents of low water content resulting very low R_F values (5). However, if capillary ascent technique recommended by Williams and Kirby (*11*) and adopted now widely is employed, the use of such solvents must be excluded, for with this technique equilibrium takes place shortly after the solvent front reaches the top of the filter paper, and the traveling of the solute ceases very soon thereafter (*12*), so that water content of the solvent affording the maximal R_F range or maximal R_F difference for the substances to be chromatographed must be employed preferentially.

The importance of controlling water content in developing solvents is further reinforced by the finding that in some cases the order of some of the separated substances can be

reserved by the change in water content of the solvent. One of the most typical examples is seen in the case of tryptophane developed with alcohols. The statement of Hanes and Isherwood (*5*) and of Bentley and Whitehead (*4*) that the water content of the solvent does not modify the order of R_F values significantly is, therefore, invalid for these cases. Whether such an example is of exceptionalna ture would be answered by further investigations.

It is also incorrect to assume that the water content of the developing solvent always increases the R_F values of substances chromatographed, although there seems to be no single exception to this rule in paper chromatography of amino acids. However, it was found that with pyronin, a basic dye, the situations are entirely reversed (*16*). Commercial pyronin was proved by paper chromatography to be a mixture of many pigments or at least to consist of several tautomeric forms of pigments of more restricted number. Chromatographed with aqueous *iso*propanol or *iso*propanol-butanol, commercial pyronin is resolved into many colored spots or streaks, the R_F values of which become lower with the increase of water contained in these solvents. Thus, with distilled water most of the pigments show little if any displacement, only very few traveling some varied distances. With alcohols of relatively low water content, most of the pigment travel, on the contrary, very significant distances. Pure alcohols have, however, failed to resolve pyronin-pigments into discrete spots; the pigments do show significant movement, but always accompanied by a considerably irregular streaking. It is now possible to resolve these pigments in a good number of dicrete spots by the way of, say, "quadriple two-dimensional chromatography": the first to forth runs are made towards the same direction from the same side of a sheet of filter paper with water and three kinds of *iso*propanol-water mixtures with decreasing water content (50, 35, and 20 per cent) in the order mentioned, and successive runs are cut off at the heights of the solvent front decreasing in the order of the runs; the fifth run is made with butanol-*iso*propanol (2:1) containing 35 per cent water in the direction rectangular to the preceding four runs. In this connection it should be mentioned that all of these pigments travel together with solvent front without any recognizable retardation when chromatographed with pehnol containing 20 per cent water.

Adenine was shown by Markham and Smith (*6*) to be somewhat similar to pyronin-pigments in its behavior to aqueous *n*-propanol: the R_F values was highest with 40 per cent water content and decreases

at each side of this concentration; thus, it decreases with the increase of water content of the solvent, if its water content is above 40 per cent.

The theory of paper chromatography developed originally by Consden, Gordon and Martin (*10*) that the distribution coefficient between two liquid phases of compounds is the direct determining factor of the R_F's obtained in paper chromatography must undoubtedly be replaced by some others, or extended to a more general form including the cases of water-miscible solvents, as repeatedly suggested (*8*, *17*) Hanes and Isherwood (*5*) as well as Bentley and Whitehead (*4*) have presented a new attractive hypothesis, although qualitative as yet, based on the experiments employing water-miscible organic solvents mixed with appropriate amounts of water as developing agents.

The hypothesis proposed assumes that substances chromatographed are adsorbed at the site of their hydrophilic groups on the postulated hydrophilic complex of water and cellulose in filter paper and that these substances travel a certain distance on the paper as a result of degree of replacement by water molecules which should compete with the molecules of substances chromatographed for the site of adsorption on the hydrophilic cellulose-water complex of filter paper.

Under the light of the findings presented here, this hypothesis would, if correct, have to be modified in such a manner, that the sites of adsorption of the solutes on filter paper are not restricted to the hydrophilic complex, but also may be located at some possible hydrophobic groups, which are competed by solutes such as pyronin or adenine and hydrophobic radicals of the solvents for adsorption, resulting a decrease of R_F values of the solutes with increase water content of solvents and an increase of R_F values by the introduction of more hydrophobic solvents as developing agents.

The knowledge on the importance of water content of developing solvents for paper chromatography would also contribute to the development of the theory of chromatography; any quantitative theory attempting to explain the movement of substances chromatographed may be requested for testing whether or not the change of R_F values effected actually by the modification of the solvent composition as regards the water content can reasonably be expected from the theory. Such an attempt was already announced preliminarily by Takanami, Kitazume and Hirota (*12*). Theories of paper chromatography would, in their ideal forms, have to contain a term of water content as a parameter.

The modification of water content in developing solvents would probably prove to be useful in the chromatography of various compounds. In this laboratory, the separation of organic acid produced through the enzymatic oxidation of homogentisic acid was carried out successfully by controlling the water content of butanol-acetic acid (4 : 1) (*18*). The chromatography of some peptides, lower and higher nucleotide polymers and lipids may become one of the most promising fields of paper chromatography, if the importance of controlling of water content in the solvents is understood more widely.

SUMMARY

1. The effect of variation in water content of developing agents for paper chromatography on the magnitude of R_F values was investigated mainly in the case of amino acids, using various organic solvents completely or partially miscible with water.

2. The R_F values of amino acids are increased with the increase of water content in a given solvent system.

3. The effect of the change of water content in a given organic solvent on the R_F values is more pronounced than the modification of other factors, such as the change of pH or the addition of some hydrophobic compounds, at least in the chromatography of amino acids.

4. The maximal R_F range of a given set of compounds does not necessarily lie at the lowest water content that affords a non-streaking separation of substances to be chromatographed.

5. In a few instances the order of magnitude of R_F values of some amino acid pairs may be reversed by the change of water content in solvents.

6. The effect of increase of water content in developing solvents does not necessarily be reflected by an increase of R_F values. With pyronin as a solute, entirely reversed situations are obtained.

7. The consequences of the results obtained were discussed in relation to practical as well as theoretical phases of paper chromatography, and the importance of controlling water content of chromatographic solvents was emphasized.

Lastly, it is our great pleasure to express our hearty thanks to Miss S. Takeda, Medico Biological Institute, Microphagen Pharmaceutical Co., Tokyo, for many suggestions and advices given to this work.

REFERENCES

(*1*) Takeda, S., and Sibatani, A., *Kagaku* (Tokyo), **21**, 92 (1951)
(*2*) Miyaki, T., Satake, K., and Hayashi, M., *Nissin Igaku* (Tokyo), **37**, 256 (1950)
(*3*) Bentley, H. R., and Whitehead, J. K., *Nature*, **164**, 182 (1949)
(*4*) Bentley, H. R., and Whitehead, J. K., *Biochem. J.*, **46**, 341 (1950)
(*5*) Hanes, C. S., and Isherwood, F. A., *Nature*, **164**, 1107 (1949)
(*6*) Markham, R., and Smith, J. D., *Biochem. J.*, **45**, 294 (1949)
(*7*) Smith, J. D., and Markham, R., *Biochem. J.*, **46**, 509 (1950)
(*8*) Stein, W. H. and Moore, S., *Cold Spring Harbor Symp, Quant, Biol.*, **14**, 179 (1950)
(*9*) Uemura, T., and Shibata, M., *J. Japan. Chem.* (Tokyo), **4**, 406 (1950)
(*10*) Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 38, 224 (1944)
(*11*) Williams, R. J., and Kirby, H., *Science*, **107**, 481 (1948)
(*12*) Takanami, M., Kitazume, Y., and Hirota, T., presented at the 7th meeting of Zoological Society of Japan, Kinki Local Section (1950)
(*13*) Jermyn, M. A., and Isherwood, F. A., *Biochem. J.*, **44**, 402 (1949)
(*14*) Magasanik, B., Vischer, E., Doniger, R., Elson, D., and Chargaff, E., *J. Biol. Chem.*, **186**, 37 (1950)
(*15*) Kawerau, E., *Biochem. J.*, **47**, xxiii (1950)
(*16*) Sibatani, A., unpublished.
(*17*) Craig, L. C., Gregory, J. D., and Barry, G. T., *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 24 (1950)
(*18*) Unpublished experiment performed in this laboratory.

The Journal of Biochemistry, Vol. 38, No. 2.

THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES. I. CONCERNING THE ETHER-INSOLUBLE LIPIDS OF LYOPHILIZED HORSE BLOOD STROMA

By TAMIO YAMAKAWA and SHIZUE SUZUKI

(*From the 7th Department of the Institute for Infectious Diseases, University of Tokyo, Tokyo*)

(Received for publication, April 9, 1951)

As compared with the remarkable progress in hemoglobin chemistry, relatively few have been reported concerning the chemical nature of erythrocyte membrane or stroma. Up to the present, Bang and Forssman (*1*), Beumer and Burger (*2*), Thannhauser and Setz (*3*), Kirk (*4*), Erickson, *et al.* (*5*), Ramsay and Stewart (*6*), Hack (*7*), Burt and Rossiter (*8*) and many other authors have reported on the lipids of stroma of various animals, but most of those works dealt mainly with the distribution of constituents and did not go beyond the quantitative determination.

Very little can be found in the literature concerning chemical nature of individual lipid. Notable exception is the classical publication of Burger and Beumer (*9*), who isolated cholesterol, cephalin and sphingomyelin from sheep blood stroma and reported the nature of these substances.

The present authors prepared a large amount of stroma samples from horse blood, extracted with organic solvents and studied each fraction. Our work concerns itself with all the component that can be isolated from the stroma. The present paper deals mainly with the extraction and fractionation of the ether-insoluble lipid constituents and with their chemical analyses. Examination of the ether-insoluble substance indicated that it was a mixture of phospholipid and sugar-containing lipid. We succeeded in separating this material into a phospholipid which was practically free from both glycerol and sugar, and a glycolipid containing only a trace of phosphorus.

By quantitative analyses of P and N, the phospholipid was proved to be a diaminomonophosphatide. On hydrolysis it gave a fatty acid and sphingosine; *i.e.*, cleavage products typical of sphingomyelin. The

fatty acid consisted exclusively of normal saturated acid belonging to the C_{16} series (palmitic acid). The purification of this phospholipid was somewhat tedious, but finally an analytically pure sphingomyelin was obtained.

Attempts were made to find, in the literature, a sphingomyelin possessing only one kind of fatty acid as its component (*10*, *11*, *12*), but all the work done proved fruitless. The component fatty acids of bram sphingomyelin were stearic, lignoceric and nervonic, and the sphingomyelin from visceral organs of mesenchymal orign (*e.g.*, spleen and lung) were found to be composed of lignoceric and palmitic acids in about equal quantities (*13*, *14*). The occurrence of a sphingomyelin with exclusively one kind of fatty acid is most unusual and, as far as we are aware, no such substance has ever been isolated.

The sugar-containing lipid gave, to our surprise, a red coloration when heated with Bial's orcinol reagent, thus indicating it might be different from ordinary cerebroside. The amount of sugar contained was about 40 per cent in contrast to 20 per cent in an ordinary cerebroside. Ehrlich's aldehyde test was also positive. When heated with mineral acid, a dark humin-like mass separated out. It resembled, in these respects, ganglioside or "Substanz X" which Klenk (*15*, *16*, *17*) previously found in the brain from a case of infantile amaurotic idiocy and later obtained from the gray matter of normal brain and cattle spleen (*18*, *19*, *20*, *21*).

The occurrence of this ganglioside-like substance in blood stroma is very unusual and, so far as we know, has not previously been reported. Despite the considerable resemblance between ganglioside and our glycolipid, it is proposed that the term "hematoside" be used to designate this substance as a matter of convenience to avoid confusion. The red coloration of Bial's orcinol reagent with blood stroma was much more intensive than with the lyophilized gray matter of human brain (*22*). As described later in detail, hematoside was hydrolyzed, yielding lignoceric acid and sphingosine, together with galactose and a water-soluble organic acid. The latter substance, on which the characteristic color reactions depend, was isolated as colorless needles. In Klenk's ganglioside (*19*), this fraction was called neuraminic acid. But in view of the analytical data, it was proved that the crystals we isolated, $C_{10}H_{23}O_{10}N$, had 2 moles of crystal-waters and the anhydrous material possessed the same molecular formula as neuraminic acid, $C_{10}H_{19}O_8N$. Since the properties of the substance we isolated seemed not quite the

same, in several respects, as those reported on neuraminic acid, we designate it hemataminic acid. This is a nitrogen-containing polyhydroxycarboxylic acid and found to reduce alkaline ferricyanide solution after hydrolysis with hydrochloric acid. The negative Trommer test of neuraminic acid reported by Klenk (*19*) and of hemataminic acid was probably due to the formation of a glycosidic linkage after methanolysis with methanolic hydrogen-chloride, thus indicating that in the original lipid it would exist in an aldehyde form, $C_9H_{17}O_7N$. This hypothetical substance was designated 'prehemataminic acid'. Whereas lignoceryl ganglioside (*21*) is composed of lignoceric acid, $C_{24}H_{48}O_2$, sphingosine, $C_{18}H_{37}O_2N$, 3 hexose, $C_{18}H_{32}O_{16}$, and neraminic acid, $C_{10}H_{19}O_9N$, minus 5 $H_2O = C_{70}H_{130}O_{21}N_2$, we propose for hematoside the following molecular formula: lignoceric acid, $C_{24}H_{48}O_2$, sphingosine, $C_{18}H_{37}O_2N$, 2 galactose, $C_{12}H_{24}O_{12}$, plus prehemataminic acid, $C_9H_{17}O_7N$, minus 4 $H_2O = C_{63}H_{115}O_{23}N_2$. The elemental composition estimated for hematoside agrees quite closely with the calculated values.

It is probable that ganglioside and neuraminic acid might be identified in future as our hematoside and hemataminic acid, respectively, but at the present stage of our investigations, it would be more justified to use these terms separately. The study of structure and configulation of prehemataminic acid is in progress, but we can assume that the substance is very likely to be derived from the condensation of hexosamine and glyceric acid following the liberation of one mole of water. The residue of stroma after thorough extraction with organic solvents, still gave the orcinol reaction as strong as the unextracted one (*22*), indicating that a large amount of this water-soluble acid remains unextracted.

The presence of ganglioside-like substance in relatively large amounts in blood would suggest that it plays an important role in this organ and it is also a matter of additional interest that no ordinary cerebroside could be isolated; in other woulds, the 'protagon' of blood stroma consists exclusively of both palmityl sphingomyelin and hematoside. Since the brain from a case of infantile amaurotic idiocy contains a large amount of ganglioside and a very small amount of cerebroside (*16*, *17*), it is perhaps reasonable to assume tnat there is something in common with each other.

EXPERIMENTAL

Preparation of the Material—The stroma samples were prepared in the following manner. The blood corpuscle paste, the residues obtained

after removal of serum in the semi-industrial production of diphtheria and tetanus antitoxins in this Institute, were treated with a large amount of saline, filtered through a cloth and centrifuged at 3,000 r.p.m. for 15 minutes. 3 *l.* of the washed, packed red cellus were added to 15 *l.* of tap water containing 50 ml. of glacial acetic acid. The supernatant hemoglobin solusion was siphoned off from the pink-colored precipitate. Washings with tap water were repeated about ten times until the washing became colorless, when the voluminous, fluffy, buff-colored stroma came up to the surface.* Washed stroma was centrifuged and further washed with distilled water. Water was squeezed out by use of dehydrating centrifuge at 4,000 r.p.m. for 30 minutes. The residual, much smaller amount of brick-colored wet mass was finally dried from frozen state under 0.1 mm. Hg. Thus, about 20-25 g. of lyophilized, pink-colored powder was obtained from 3 *l.* of packed red cell. During the course of this study in a year, more than 2 kg. of lyophilized stroma was obtained.

Extraction of Lipids—All the solvents used were carefully purified and and freshly distilled. 200 g. of lyophilized stroma was ground to a fine powder in a porcelain mill and extracted at room temperature with a mixture of equal parts of peroxide-free anhydrous ether and aldehyde-free anhydrous methanol for 6 days with occasional shaking;—700 ml. of the solvent being used for every extraction. Four such extractions were carried out and the residues were filtered on Buchner funnel each time and washed thoroughly with the same solvent. Each extract was evaporated nearly to dryness under reduced pressure, at a temperature not exceeding 50°C. The combined extract yielded a large amount of crystals of cholesterol. After removal of cholesterol by suction and the filtrate further concentrated *in vacuo*, the residual light yellowish syrup was dissolved in a small amount of anhydrous ether and was placed overnight in the icebox. A white amorphous precipitate which separated was centrifuged off and washed with ether. The supernatant yellowish liquid was concentrated to a small volume and the phosphatide was precipitated with acetone. The phosphatide was redissolved in anhydrous ether and cooling gave an additional amount of precipitate which was collected, washed with ether, and combined with the first lot. For the purpose of obtaining the white precipitate, cooling in the centrifuge-tube with dry-ice-acetone mixture following centrifuging was highly satisfactory. The

* Erickson, *et al.* (*5*) found that washing procedures did not remove measurable amounts of lipid from the stroma.

washed precipitate showed a waxy appearance. The ethereal solution and washings were combined and reserved for further investigations.

The ether-methanol treated residue was air-dried and exhaustively exteacted in a Soxhlet apparatus with the mixture of an equal amount of chloroform and methanol. The extract was intensely dark-brown in color.* Extraction was continued for about 40 hours. The extract was evaporated under reduced pressure and the almost resinous, dark-brown residue was dissolved in a small volume of chloroform and precipitated with a large excess of acetone. By centrifuging, the darkbrown color transited to the supernatant acetone and the precipitate became nearly colorless. After repeating the procedure once more, the precipitate was recrystallized from a large amount of boiling absolute alcohol containing a small volume of chloroform. The crude substance was light gray, friable, reasonably stable pywder.

Yield of Crude Materials—Cholesterol, 3-6 per cent; ether-soluble lipid, 3-6 per cent; ether-insoluble portion of ether-methanol extract, 1-2.5 per cent; chloroform-methanol extract, 1-2.5 per cent, of dry stroma.

Analytical Methods—Phosphorus was estimated by Allen's method (*23*), iodine number by Yasuda's (*24*) and glycerol by Blix' (*25*). Dry combustion methods were used for the estimation of C, H and N. Since ceric sulfate is not available at present, Hagedorn-Jensen method for sugar estimation, modified by Wierzuckowski, *et al.* (*26*), was used. Particular consideration was paid in order to avoid the probable error due to the unsaturated compound in the lipid. The amount of sugar reported in this paper was calculated as galactose in every case.

Purification of the Ether-insoluble Lipids—11.5 g. of the ether-insoluble portion of the ether-methanol extract (P 2.80) was dissolved in warm pyridine in a centrifuge-tube and cooled at room temperature. The almost white amorphous solid which appeared gelatinous was centrifuged. The supernatant light yellowish fluid was decanted off and the pyridine procedure was repeated once more. The supernatant pyridine was combined with the first crop. The rediment was washed with acetone and ether and dried *in vacuo*. (*Sph. A.*, 8.1 g., P 3.95, N 3.44, glycerol 2.74, sugar 5.4). The supernatant pyridine solution was passed through an alumina-column in order to remove the phospholipid. The filtrate was evaporated *in vacuo* and precipitated with a large excess of acetone.

* In the case of a brain, by the same treatment, the extract remains nearly colorless, or at most, straw-colored.

Yield, 1.25 g., P 0.95, sugar 18.3.

An attempt was made to separate the components by the procedure recommended by Thannhauser, *et al.* (*14*). 8 g. of *Sph. A.* was dissolved in 10 volumes of warm glacial acetic acid and allowed to cool in the refrigerator overnight. The precipitate that separated was filtered off and washed with acetone and ether; 1.9 g. The phospholipid was rdcovered from the concentrated filtrate by precipitation with acetone (*Sph. B.*, 5.6 g., P 3.88, sugar 4.07).

In order to remove the sugar.containing lipid, 5.5 g. of *Sph. A.* was dissolved in a mixture of ethanol-benzin (1 : 9) and the solution was put through a chromatographic column of alumina. The faintly turbid filtrate was concentrated *in vacuo* and precipitated with a large amount of acetone. (*Sph. C.* 3.2 g., P 3.92, sugar 4.52, glycerol 0.48.).

The pooled acetone and ether washings were combined and concentrated to dryness and precipitated with a large excess of acetone. P. 3.92, glycerol 3.70. (Hydrolecithine rich fraction).

6.3 g. of *Sph. C.* was dissolved in 100 ml. of absolute ethanol-glacial acetic acid (9 : 1) and passed through a 10 cm.-long alumina column for the selective adsorption of sugar-containing lipid, and the column was washed out with the same solvent. The filtrate and washings were combined and cooled in the icebox. After removal of the precipitate which separated out, the solution was passed through a freshly prepared alumina column. The fiiltrate, along with the washings, were cooled, the precipitate formed contrifuged off, the supernatant evaporated to near dryness *in vacuo*, precipitated with a large amount of acetone and washed with ether. The dry residue weighed 4.9 g. (*Sph. D.*, sugar 2.11, glycerol 0.24). 5.0 g. of *Sph. D.* was recrystallized from hot ethyl acetate. After cooling very slowly, a fine, colorless deposit was obtained, but macroscopically, it showed no definite crystalline structure. Only by microscopic observation, a sphaerolytic crystal was detected. The substance is easily soluble in benzene, chloroform, soluble in warm ethanol, warm pyridine and hot ethyl acetate. Heated in a capillary tube, the substance sintered at about 90°, turned yellow at 200-210° and melted to a clear fluid at 215-7°. (*Sph. E.*, 3.72 g.).

Optical Rotation—0.3987 g. of the substance dissolved in chloroform-methanol (1 : 1) to 10 ml. gave, in a 1 dm. tube, $\alpha_D = +0.28°$, hence $[\alpha]_D^{10} = +7.04°$.

Analyses—Found: C 64.63, H 11.35, N 3.89, P 4.27, sugar 1.71, iodine number 30.4.

Calculated for palmityl-sphingomyelin ($C_{31}H_{61}N_2PO_7$, mol. wt., 720.71): C 64.76, H 11.19, N 3.86, P 4.31, iodine number 35.2.

Hydrolysis of Sphingomyelin—3.00 g. of *Sph. E* was added to a solution of 10 ml. of conc. sulfuric acid in 90 ml. of methanol and the mixture was heated on a water-bath under a reflux for 8 hours. During the hydrolysis, no coloration occurred. After cooling, the reaction product was extracted with successive portions of petroleum ether. The petroleum ether extract was washed with water, dried over sodium sulfate, filtered and evaporated to dryness. To the methanolic solution was added an aqueous solution of barium hydroxide until the reaction was alkaline to phenol-phthalein. After the precipitated barium sulfate was removed, the solution was extracted three times with ether to obtain the free base.

Separation of Fatty Acid—The methyl ester of fatty acid which was obtained on evaporation of the petroleum ether extract was almost colorless and weighed 0.98 g., representing 33 per cent of the phosphatide. The substance was fractionated by distillation into the following portions: b.p. 106-120°/0.03, 0.60 g., m.p. 26.0-26.5°; b.p. 130-150°/0.03, 0.18 g., m.p. 28-9°; residue in the flask, 0.18 g., m.p. 25-37°. The slightly brown residue in the flask was recrystallized from methanol to colorless plates melting at 46-7°.

2nd Trial—2.00 g. of sphingomyelin, containing 4.15 per cent of P and 1.74 per cent of sugar, gave, on hyrolysis, 0.79 g. of fatty acid methyl ester, which was fractionated as follows:

b.p. 129–132°/0.25, 0.27 g., m.p. 26.5–27°, iodine number 1.32;
b.p. 133–135°/0.25, 0.25 g., m.p. 25–26°, iodine number 4.13;
b.p. 140–175°/0.25, 0.12 g., m.p. 40–44°, iodine number 27.9.

From the last fraction, 33 mg. of colorless plated, m.p. 49-52°, was obtained (this is perhaps an impure methyl lignocerate). All the fractions except the last one, were combined, weighing 1.40 g., and was converted to the free acid by saponification with methanolic potassium hydroxide. The free acid, recrystallized from petroleum ether, melted at 60°, and showed no depression of the melting point when fused with the authentic sample of palmitic acid, melting at 63°.

Calc. for palmitic acid: COOH 17.56. Foucd: 17.60.

Base Fraction—Light brown-colored free base, weighing 0.65 g., was dissolved in warm petroleum ether. The solic that separated was dried and acetylated by warming with 1 ml. of acetic anhydride in 1 ml. of pyridine. The crystalline acetate which separated was recrystallized

from acetone to colorless needles, melting at 98-100°. $[\alpha]_D^{12} = -9.6°$ (0.1 g. in 10 ml. of chloroform).

Calcd. for triacetylsphingosine, $C_{24}H_{43}O_5N$: C 67.76, H 10.12, N 3.29.

Found: C 67.61, H 10.15, N 3.62, iodine number 46.7.

Purification of Chloroform-Methanol Extract (Hematoside)—The chloroform-methanol extract contained a large amount of sugar and a small amount of phosphorus, suggesting it was a glycolipid. In order to remove the phosphorus-containing impurities, 8.3 g. of the gray-white powder was recrystallized from glacial acetic acid. Adsorption on alumina with a hot pyridine solution was also applied. Finally, 3.2 g. of the colorless powder was obtained. In this case, too, no definite crystalline structure could be observed, but the analytical values indicated that the substance was extremely pure. Heated in a capillary tube, the substance sintered at 90°, turned yellow at 170°, darkened at 190° and melted with decomposition at 225-7°.

Optical Rotation—1.00 g. of the substance dissolved in pyridine and diluted to 10 ml., gave, in a 1 dm. tube, $\alpha_D = -1.19°$, hence $[\alpha]_D^{15} = -11.9°$.

Analyses—Found: C 61.72, 61.91, H 9.64, 9.80, N 2.27, 2.28, P 0.06, 0.18, sugar 38-40, iodine number 19.0, COOH 4.19, 3.66.

Calcd. for $C_{53}H_{118}O_{21}N_2$ (1223.59): C 61.84, H 9.72, N 2.29, iodine number 20.7, COOH 3.68.

Hydrolysis of Hematoside—

Fatty Acid Fraction—4.0 g. of hematoside was dissolved in 80 ml. of 8 per cent methanolic hydrochloric acid in a sealed tube and heated for 3 hours in a boiling water-bath. At the end of hyrolysis, the mixture darkened to some degrees and on cooling, a large amount of crystals, due to the methyl ester of the fatty acid, separated out. The content of the tube was extracted with petroleum ether, which after washing with water, was dried over sodium sulfate, filtered, and evaporated to drpness. The residue weighed 1.20 g. and was solid at room temperature, which was recrystallized from methanol to colorless plates, melting at 57.5-58.5°. In another trial, the methyl ester was distilled at a pressure of 0.015 mm. The distillation of the material covered a boiling range between 160-168°. The yield of the methyl ester was 28.4 per cent of the lipid. It was converted to the free acid by saponification with methanolic potassium hydroxide. The free acid, recrystallized to colorless plates from glacial acetic acid, melting at 79°, and showed no depression of the melting point when fused with the authentic sample of lignoceric acid, m.p. 83°.

Calcd. for lignoceric acid: COOH 12.24. Found: 12.29.

Base Fraction—After extraction with petroleum ether, methanol was evaporated under reduced pressure, fresh portions of methanol were added and evaporation was repeated in order to remove hydrochloric acid. Lead carbonate was added and lead chloride filtered off. The filtrate was made alkaline to phenolphthalein with barium hydroxide and extracted three times with ether. The ethereal extract was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness, leaving 0.93 g. of the crude base as a light brown solid. This was dissolved in warm petroleum ether and separated into 0.21 g. of insoluble white precipitate and 0.71 g. of soluble brown solid. The insoluble substance was acetylated with pyridine and acetic anhydride. The acetate was recrystallized from acetone to colorless needles, melting at 101-102.5°. $[\alpha]_D^{20} = -5.9°$ (50.91 mg. in 2 ml. of chloroform).

Calcd. for triacetyl-sphingosine: $C_{24}H_{43}O_5N$: C 67.76, H 10.12, N 3.29.

Found: C 67.54, H 10.42, N 3.28, iodine number 44.0

Sugar Fraction—After extraction with ether, the aqueous solution was saturated with carbon dioxide to remove the excess of barium hydroxide, silver sulfate added until no further precipitate separated, after which the precipitated silver chloride and barium sulfate were filtered off, and the solution was decomposed with hydrogen sulfide. The filtrate was made alkaline with barium hydroxide and carbon dioxide passed through the solution. The solution obtained after filtration was evaporated nearly to dryness under reduced pressure. To the residue was added 2 ml. of methanol and the solution was diluted with about 50 ml. of acetone, whereupon a white, flocculent, fluffy precipitate appeared, which was collected, washed with acetone and immediately dried in a vacuum desiccator, for it was strongly hygroscopic. This material gave red coloration with Bial's orcinol reagent and was examined, as will be described later.

The acetone solution was evaporated to dryness *in vacuo* and dried in a vacuum desiccator and the residue weighed 1.07 g., representing 27 per cent of the lipid. The reaction with Bial's reagent was negative. The light yellowish syrup was heated with 2 ml. of 5 *N* hydrochloric acid for 1 hour on a steam bath and diluted with distilled water. Lead carbynate was added to remove hydrochloric acid, the excess of lead precipitated with hydrogen sulfide and the solution was treated with charcoal. The perfectly clear aqueous solution gave, in a 2 dm. tube, $\alpha_D = +4.41°$. 1 ml. of this solution was diluted to 50 ml. and the sugar

content was estimated with 1 ml. of this solution. 0.543 mg. of sugar (calcd. as galactose) was found to be contained. Hence the solution used for estimation of optical rotation contained 2.72 per cent of the sugar.

$$\text{Therefore, } [\alpha]_D^{10} = \frac{+4.41 \times 100}{2.72 \times 2} = +81.1°.$$

Since the specific rotation of D-galactose is +81°, the sugar in hematoside seemed to be D-galactose. The aqueous sugar solution was concentrated to about 2 ml., 2 ml. of glacial acetic acid and 0.5 g. of α-methyl-phenyl-hydrazine were added. Light yellowish powder which separated was twice recrystallized from methanol to colorless plates, m.p. 187-9°. There was no depression of the melting point on admixture with pure D-galactose α-methylphenylhydrazone, m.p. 189°.

Water-soluble Acid Fraction (*Hemataminic Acid*)—The colorless, acetone-insoluble precipitate, weighing 0.42 g., which oonsisted of the barium salt of an organic acid, was dissolved in 10 ml. of distilled water and barium was removed quantitatively with dilute sulfuric acid. The colorless filtrate was concentrated carefully *in vacuo* to a volume of about 20 ml. 10 ml. of absolute ethanol and 5 ml. of ether were added and allowed to stand overnight in the refrigerator. Colorless needles which separated was collected, weighing 159 mg. For analysis, the substance was dried at 50° *in vacuo* over P_2O_5:

Calcd. for $C_{17}H_{23}O_{11}N$ (317.29): C 37.86, H 7.31, N 4.41.
Found: C 38.20, H 7.66, N 4.39.
Dried over P_2O_5 at 80° for 1 hour:
Calcd. for $C_{17}H_{21}O_{10}N$ (299.28): C 40.13, H 7.01.
Found: C 40.32, H 7.05.
Dried over P_2O_5 at 120° for 5 hours:
Calcd. for $C_{11}H_{11}O_7N$ (281.26): C 42.68, H 6.81, N 4.98.
Found: C 42.41, H 6.54, N 4.95.

The substance free from crystal-water, designated tentatively as hemataminic acid, was highly hygroscopic. It gave positive reactions with Bial's orcinol, Ehrlich's aldehyde and vanillin hydrochloric acid reagents as well. Molisch reaction was negative. Ninhydrin test was negative* but became positive in the presence of alkali. Heated under a microscope, the hpdrous, transparent, glassy needle became white at 100-110° by dehydration, turned somewhat brownish at about 180°, brown at 205°, finally changed into a black charcoal retaining the

* Ninhydrin reaction on filter paper was positive.

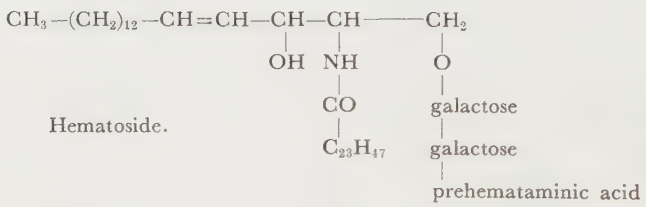
original arystal shape, at 220°. It did not reduce alkaline ferricyanide solution, but became able to reduce it after hydrolysis with hydrochloric acid. The reducing power corresponded to ca. 55 per cent of that of galactose. The substance was investigated as will be reported in a separate paper.

DISCUSSION

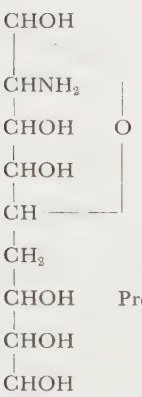
The occurrence of orcinol-positive lipid was first noticed by Walz (*27*), while studying the cerebroside of a bovine spleen. Later, Klenk (*15*) found that the lipid of the brain from a case of infantile amaurotic idiocy (Tay-Sachs' type) was composed of a large amount of sphingomyelin and 'Substanz X', and ordinary cerebroside was almost nonexistent. Substanz X, which was responsible for the orcinol reaction, was later purified and the sugar in the sample amounted to 31.7 per cent. Its elemental composition was also reported as C 59.15, 58.78, H 9.34, 9.30, N 2.52 (*16*). Orcinol-positive substance was also isolated by Blix (*28*) from the protagon of bovine brain and the presence of hexosamine was noted. In a subsequent paper (*17*), Klenk obtaine da less pure sample and reported that it gave positive ninhydrin reaction. In the same year, he prepared the glycolipid from the cerebroside fraction of normal brain, and isolated a water-soluble crystalline polyhydroxy-amino acid as a component. This was named neuraminic acid (*18*). It gave positive reactions with ninhydrin and orcinol, as well as Ehrlich's aldehyde reagent. It seems that he named the lipid as ganglioside and also isolated it from bovine spleen (*20*, *21*). The original reports of the latter two were unprocurable in Japan and present, but according to the summarized descriptions in the Chemical Abstracts, ganglioside containing 39.4 per cent of sugar is composed of fatty acid, sphingosine, hexose and neuraminic acid in a ratio of 1 : 1 : 3 : 1. He proposed for lignoceryl ganglioside the molecular composition, $C_{70}H_{130}O_{26}N_2$. Accordingly, the calculated values are: C 59.42, H 9.12, N 1.97, sugar 38.2.

Hematoside, the glycolipic we found in the blood stroma resembled ganglioside in many respects. But the C, H and N values and the amounts of methyl lignocerate and sphingosine is olated from hematoside were somewhat higher than those calculated from Klenk's assumption. The yield of sugar fraction ioslated was, on the contrary, too low in every trial. Whereas Klenk reported that neuraminic acid was Trommer-negative and ninhydrin-positive, hemataminic acid, the amino acid fraction in our lipid, acquires reducing power after hydrolysis with hydro-

chloric acid and is ninhydrin-positive only in the presence of a trace of alkali. This behaviour towards ninhydrin reagent resembles quite closely with that of glucosamine hydrochloride.* Klenk reported that the crystal-water of neuraminic acid was easily liberated by drying the sample at 50° *in vacuo*, but our sample still contains 2 moles of crystal-waters at this condition and does not stay at constant weight until it is dried at 120° *in vacuo* for a long time. It is likely that hemataminic acid is an artefact through cleavage with methanolic hydrochloric acid and is the methyl ether of, so to speak, prehemataminic acid in a glycosidic linkage. The assumption of the presence of an aldehyde group in prehemataminic acid is very favorable in order to explain the combination of the components in the lipid. It is also consistent with the higher amount of sugar determined by the reducing power if two moles of hexoses are contained in it. The sugar contained in hematoside was proved to be exclusively D-galactose and no glucose was detected, al-



Hematoside.



(supposed fomulae)

Prehemataminic acid.

* In this connection, we wish to thank Prof. H. Masamune for suggestion.

though Klenk reported that the sugars of ganglioside were composed of galactose and glucose in a ratio of 3:2 (*20*), and in another report, in a ratio of 2:1 (*29*). In view of optical rotation and iodine number, the base fractions of both hematoside and sphingomyelin are composed of sphingosine, and no dihydro-compound seems to be present in any appreciable amount (Cf. Carter, *et al.* (*30*)).

Comparing all these facts, we propose for hematoside the molecular formula, $C_{63}H_{113}O_{20}N_2$, *i.e.*, lignoceric acid, sphingosine, glactose and prehemataminic acid, in a ratio of 1:1:2:1. Judged from the present results, we assume the structure of hematoside as follows: 2 mole of galactose and prehemataminic acid combine with the terminal hydroxyl group of lignoceryl sphingosine, setting the carboxylic group of prehemataminic acid free. The exact formula of hemataminic acid cannot yet be established from the available data but in view of the present results, the structure similar to glucosamine or chondrosamine likely to be present as its part.

The authors wish to express their gratitude to Prof. S. Akiya, Dr. T. Ukita and Dr. H. Yoshikawa for encouragement, criticism and advice. They also thank Miss R. Ota and Mr. B. Kurihara for C, H and N analyses.

SUMMARY

1. Two kg. of lyophilized horse blood stroma was prepared and exracted with organic solvents.

2. From the ether-insoluble lipid fraction, palmityl-sphingomyelin and a glycolipid, which is described under the name of hematoside, were isolated.

3. The cleavage products from both lipids were studied in detail.

Addendum: 1. After this paper was submitted, Prof. Dr. E. Klenk has the kindness to send us his detailed papers. According to the report (*21*), he already noticed that neuraminic acid in paper (*19*) possessed a methoxyl group. He used, thereafter, the term neuraminic acid, $C_{10}H_{19}O_9N$, for the non-methoxyl substance which corresponds our prehematamidic acid. Whereas he prefer for the methoxyl-containing acid $C_{11}H_{21}O_9N$ to $C_{10}H_{19}O_8N$, we take the latter for our hemataminic acid.

2. Though the structural study of hematoside is being continued, it is justified to state that the amino group of prehemataminic acid in the original undegrated lipid is probably substituted by an acyl group, since no amino nitrogen is found in in hematoside by Van Slyke's method.

REFERENCES

(1) Bang, I., and Forssman, J., *Beitr. chem. Physiol. Path.*, **8**, 238 (1906)
(2) Beumer, H., and Bürger, M., *Arch. exp. Path. Pharm.*, **71**, 311 (1913)
(3) Thannhauser, S. J., and Setz, P., *J. Biol. Chem.*, **115**, 533 (1936)
(4) Kirk, E., *J. Biol. Chem.*, **123**, 623 (1938)
(5) Erickson, B. H., Williams, H. H. Bernstein, S. S., Avrin, I., Jones, R. L., and Macy, I. G., *J. Biol. Chem.*, **122**, 515 (1938)
(6) Ramsay, W. N. M., and Stewart, C. P., *Biochem. J.*, **35**, 39 (1941)
(7) Hack, M. H., *J. Biol. Chem.*, **169**, 137 (1947)
(8) Burt, N. S., and Rossiter, R. J., *Biochem. J.*, **46**, 569 (1950)
(9) Bürger, M., and Beumer, H., *Biochem. Z.*, **56**, 446 (1913)
(10) Merz, W., *Z. physiol. Chem.*, **193**, 59 (1930)
(11) Fränkel, E., Bielschowsky, F., and Thannhauser, S. J., *Z. physiol. Chem.*, **218**, 1 (1933)
(12) Thannhauser, S. J., and Benotti, J., *Z. physiol. Chem.*, **253**, 217 (1938)
(13) Thannhauser, S. J., Benotti, J., and Boncoddo, N. F., *J. Biol. Chem.*, **166**, 677 (1964)
(14) Thannhauser, S. J., and Boncoddo, N. F., *J. Biol. Chem.*, **172**, 141 (1948)
(15) Klenk, E., *Z. physiol. Chem.*, **235**, 24 (1935)
(16) Klenk, E., *Z. physiol. Chem.*, **262**, 128 (1939-40)
(17) Klenk, E., *Z. physiol. Chem.*, **267**, 128 (1941)
(18) Klenk, E., and Langerbeins, H., *Z. physiol. Chem.*, **270**, 185 (1941)
(19) Klenk, E., *Z. physiol. Chem.*, **268**, 50 (1941)
(20) Klenk, E., ank Rennkamp, F., *Z. physiol., Chem.*, **273**, 253 (1942); *Chem. Abstr.*, **37**, 5743 (1943)
(21) Klenk, E., *Z. physiol. Chem.*, **273**, 76 (1942); *Chem. Abst.*, **37**, 6285 (1943)
(22) Unpublished.
(23) Allen, R. J. L., *Biochem. J.*, **34**, 868 (1940)
(24) Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931-2)
(25) Blix, G., *Mikrochim. Acta*, **1**, 75 (1937)
(26) Wierzuckowski, M., Dzisiow, F., Sysa, J., and Borkowski, Z.,

Z. physiol. Chem., **253**, 231 (1938)
(*27*) Walz, E., *Z. physiol. Chem.*, **166**, 210 (1927)
(*28*) Blix, G., *Skand. Arch. Physiol.*, **89**, 46 (1938)
(*29*) Klenk, E., and Schumann, E., *Ber. chem. Ges.*, **75B**, 1632 (1942); *Chem. Abst.*, **39**, 1559 (1945)
(*30*) Carter, H. E., Norris, W. P., Glick, F. J., Phillips, G. E., and Harris, I., *J. Biol, Chem.*, **170**, 269 (1947)